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DEVELOPMENT OF TEST FOR DETERMINING ANAEROBIC BIODEGRADATION POTENTIAL

by

Daniel R. Shelton and James M. Tiedje
Department Crop and Soil Science
Michigan State University
East Lansing, MI 48824

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Task Officer
Robert H. Brink
Exposure Evaluation Division
Office of Toxic Substances
Washington, DC 20406

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16. Abstract (Limit: 200 words)

This project was for the purpose of developing and evaluating a suitable screening-level laboratory method for the evaluation of the potential that an organic compound might be susceptible to anaerobic biodegradation (methanogenesis) in the environment. As it developed, the project focused on a method under consideration by the American Society for Testing and Materials (ASTM) and the report contains several recommendations as appropriate, for the modification of the draft ASTM method. Overall, the report, with the recommended modifications, consists of a single-lab validation of the basic method. The method also appears as one of the EPA Office of Toxic Substances Chemical Fate Test Guidelines.

In the method, a chemically defined anaerobic medium containing resazurin, as an oxidation/reduction indicator, and 10% primary anaerobic digestor sludge are dispensed in 100-mL portions into serum bottles. Selected bottles are supplemented with test substance at a concentration equivalent to 50 mg/L as organic carbon. Gas production is measured at appropriate intervals. The extent of biodegradation is determined by comparing gas production from blank control bottles and bottles containing the test substance.

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Preface

The goal of this research was the development and validation of a screening level test for determining anaerobic biodegradation potential. The requirements of such a screening level test are that the method be easily and rapidly performed with a minimum of equipment, that the results be reliable and easily interpreted, and that the method be able to accomodate a wide variety of chemical substances, This necessarily eliminates the use of more sophisticated analytical techniques which, although more definitive, are too costly and too specific. Measurement of ultimate biodegradation or mineralization is appropriate for such a screening method because the products of mineralization (CH_4 and CO_2 in anaerobic habitats) can be easily monitored and are independent of parent chemical structure. This is also desirable in that only compounds which are mineralized will give positive results; it is unlikely that compounds which readily undergo mineralization will pose a significant environmental threat.

We have conducted experiments which we feel adequately define and validate the significant test parameters of this method. Based on the results of these experiments and our experience with the method, we feel that the proposed method will yield consistent and reliable results. The salient features of the recommended test method are given below.

1) A 10% sludge inoculum consisting of primary anaerobic sludge with 15-30 day retention time and total organic solids of approximately 1.0-2.0% is recommended. Sludges can be stored for up to 4 weeks at 4°C in tightly capped containers; however, fresh sludge should be used whenever possible.

2) Any standard anaerobic mineral salts medium with a 2 mM phosphate buffer and 1.2 g $NaHCO_3$/liter with a 10% CO_2/90% N_2 headspace is recommended. Sulphide can be added to ensure reducing conditions in concentrations not to exceed 1 mM. Since active sludge has a strong O_2 consuming capacity we have not found this necessary.

3) Chemicals should be added to achieve a final concentration of 50 ppm carbon. Chemicals should be introduced into test bottles, prior to addition of 10% sludge, in one of four ways depending on phase and solubility: (1) liquids should be dispensed into bottles with a microsyringe, (2) water soluble solids should be dissolved in water, then dispensed into bottles, (3) water insoluble solids should be dissolved in di-ethylether (or comparable solvent), the ether solution dispensed into bottles and then allowed to evaporate for at least 2 hours, and (4) insoluble polymers should be weighed out and added to bottles.

4) One hundred milliliters of 10% sludge solution in 160 ml serum bottles with new butyl rubber stoppers should be used.

5) Serum bottles should be incubated at 35°C for at least 8 weeks or until biodegradation is complete. If gas production is still in progress at the eighth week, then incubations should be continued until gas production is complete.

6) Gas production should be measured using a pressure transducer with multimeter which is connected to the test bottle with a 3-way valve and 20-gauge needle. Measurements should be made on a weekly basis; excess gas pressure should be vented.

7) Reference chemical(s) should be included with each series of incubations in order to characterize sludge activity. p-Cresol and/or phthalic acid are suggested as a more rigorous test of biodegradation capacity. Ethanol, which is easily degraded, can be used as a positive control.

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Abstract

This project has been for the purpose of developing and evaluating a suitable screening-level laboratory method for the evaluation of the potential that an organic compound might be susceptible to anaerobic biodegradation in the environment. As it developed, the project focused on an evaluation of a method under consideration by the American Society for Testing and Materials (ASTM) and the report contains several recommendations, as appropriate, for modifications to the ASTM method. Overall, the report, with the recommended modifications, consists of a single-lab validation of the basic method. The method will be presented as one of the TSCA guideline methods for citation in chemical-specific test rules. The method also has been recommended for consideration in the updating process of the Organization for Economic Cooperation and Development (OECD).

In the method, a chemically defined anaerobic medium containing resazurin as an oxidation/reduction indicator and 10% primary anaerobic disgestor sludge are dispensed in 100-ml portions into serum bottles. Selected bottles are supplemented with test substance at a concentration equivalent to 50 mg/l as organic carbon. Gas production is measured with a pressure transducer. The extent of biodegradation is determined by comparing gas production from blank control bottles and bottles containing the test substance.

Introduction

The Toxic Substance Control Act was passed with the intent of preventing new chemical products from posing an unreasonable risk to health or to the environment. In order to assess such risks, a knowledge of the fate of chemicals in the environment, including whether or not degradation occurs, is necessary. Hydrolytic, photochemical, and aerobic microbial degradation are processes which have been fairly well studied and for which reasonable test methods exist. This is not the case for anaerobic biodegradation; no proven methods for a generalized test exist in the scientific literature. The purpose of this project was the development and validation of a general screening method for determining the anaerobic biodegradation potential of organic compounds.

Background

Few studies have been conducted in the past on the anaerobic biodegradation of organic compounds of industrial or environmental significance. Earlier work focused primarily on the metabolism of model substrates such as benzoic acid, using sludge as an inoculum (2,5,6,10,15,18), or the transformation of pesticides in flooded soils (1,13,20,23,24). None of these studies provided the necessary experimental framework for describing a general anaerobic biodegradation test method. Recently, Healy and Young have described the anaerobic biodegradation of several hydroxy and/or methoxy substituted benzoic acids and substituted phenols (7,8). They used a 10% sludge inoculum with mineral salts medium and assessed ultimate biodegradation (mineralization) by measuring gas production. Based on the work of Healy and Young, a committee of the Amer. Soc. for Testing Materials (ASTM) has proposed a test method. The salient features of the ASTM method are: (1) a 4 week incubation period, (2) a test compound concentration of 50 ppm carbon, (3) slightly modified Healy and Young medium and headspace of 30%-70% CO_2/N_2, (4) introduction of water-insoluble materials into sludge bottles by disolving them in dioxane, and (5) assessment of biodegradation by measuring gas production using a pressure transducer (with multimeter). We have used the proposed ASTM method as a starting point for our own studies in modifying, refining, and/or validating the significant test parameters.

Survey of organic compounds for anaerobic biodegradation (compound survey)

As previously stated, there is little information in the literature concerning the anaerobic biodegradation of synthetic organic chemicals (xenobiotics). One of our first priorities was to conduct a biodegradation survey of a variety of organic chemicals with the following goals in mind: (1) attempt to establish a structure vs. biodegradability scheme, (2) use the results to select compounds for future methods testing, (3) gain experience handling xenobiotics with different physical properties and working with anaerobic sewage sludge, and (4) assess the appropriateness of a 10% sludge inoculum.

Secondary anaerobic sewage sludge from Adrian and Jackson waste treatment plants was collected in 1 liter jars, tightly capped, and stored at 4°C until use (fresh sludge was collected once a month). A 10% sludge inoculum was prepared by adding sludge, filtered through one layer of cheesecloth, to 1-2 liters of mineral salts medium consisting of (per liter) 0.30 g KH_2PO_4, 0.35 g K_2HPO_4, 0.5 g NH_4Cl, 0.1 g $MgCl_2 \cdot 6H_2O$, 70 mg $CaCl_2 \cdot 2H_2O$, 20 mg $FeCl_2 \cdot 4H_2O$, 1 ml of a trace metals solution (25), 1.2 mg $NaHCO_3$, and 120 mg $Na_2S \cdot 9H_2O$, which had been autoclaved (to drive off O_2), then cooled to approximately 35°C while sparging with a 10% CO_2-90% N_2 gas mixture. One hundred milliliter aliquots of 10% sludge were dispensed into 160 ml serum bottles (in the Wheaton catalogue these are listed as 125 ml bottles) while sparging with a 10% CO_2-90% N_2 gas mixture. Test bottles had previously been amended with 50 ppm test compound as follows: liquids were dispensed via syringe, water-soluble solids were dissolved in water, then dispensed, while water-insoluble solids were dissolved in diethylether, dispensed, and the ether allowed to evaporate (1-2 hours). Bottles were incubated at 35°C from 4 to 14 weeks. All compounds were tested in duplicate. Methane production was monitored weeky; if methane production was observed in test bottles over controls (no chemical added), those bottles were again amended with substrate as before, except that water-insoluble solids were dissolved in dioxane to facilitate additions. Methane was quantified using a FID equipped gas chromatograph with a 2 m Tenax-GC column; the carrier gas flow rate was 30-40 ml/min N_2. Degradation is expressed as percent theoretical methane production based on the stoichiometry of mineralization (see Appendix I for calculations). Lag

time represents the minimum time period (in weeks) before significant methane production over controls was observed.

A total of 98 compounds were tested. Thirty-nine were degraded to some extent (in at least one sludge) (Figure 1), 49 were neither degraded nor inhibitory (Figure 2), and 10 were inhibitory (Figure 3). Based on these data, it is difficult to make generalizations regarding structure vs. biodegradability. Of the substituted phenols, m- and p-methylphenol were degraded, chloro- and aminophenols were not, while of the substituted benzoates none of the methylbenzoates were degraded but several chloro- and aminobenzoates were; of the chlorobenzoates, only m-chlorobenzoate was degraded, while of the aminobenzoates, m-aminobenzoate was the only one not degraded. Also, 2-octanol was degraded while 2-hexanol was not; benzyl alcohol and 4-hydroxybenzyl alcohol were degraded while 2-hydroxybenzyl alcohol was not. It does appear that the sole presence of hydroxy and/or carboxy substituents (regardless of number or position) usually confers biodegradability on the aromatic nucleus. Although we feel confident of the data indicating biodegradability, we cannot be certain that those compounds for which we found no evidence of biodegradation are, in fact, persistent. This is based, in part, on the observation that degradation results for a few compounds which were particularly difficult to degrade (long lag times) were not consistent. For example, 4-hydroxybenzyl alcohol was not degraded initially, however, when retested, biodegradation was apparent by the 8th week (Jackson sludge only); p-aminobenzoate and m-chlorobenzoate acid were degraded initially in both Adrian and Jackson sludges, however, when retested, m-chlorobenzoate was not degraded and p-aminobenzoate was degraded in Jackson sludge only. After gaining experience with sludges we now believe that secondary anaerobic sludge, such as used in the survey experiment, does not have the metabolic diversity and activity of primary anaerobic sludge (see Appendix II), particularly when used as a source of inoculum up to 30 days after collection. Early length of storage experiments with secondary anaerobic sludge indicated that a 30 day storage period was not detrimental to the degradation of glucose, ethanol, propionic acid, or butyric acid. However, we now question whether degradation results for easily degraded substrates such as glucose, etc. can be extrapolated to compounds that are more difficult to degrade. Considering that Adrian and Jackson secondary anaerobic sludges are unmixed and unheated (these experiments were

conducted during the summer months) with retention times of 60-90 days, it seems almost certain that some loss of viability would have occurred, particularly after a 30 day storage period. Since serum bottles were generally discarded after 8 weeks of incubation in the absence of net methane production, any extension of the lag time beyond 8 weeks would have been regarded as lack of degradation.

Comparison of parent compound disappearance in whole sludge vs. mineralization in 10% sludge

Since the purpose of the test method is to accurately assess anaerobic biodegradation potential of xenobiotics, it is important that biodegradation results obtained with a 10% sludge inoculum be consistent with or representative of the fate of xenobiotics in actual anaerobic habitats[1]. In particular, we felt it important to establish the degree of correlation between degradation in 10% sludge vs. whole sludge for selected compounds. Compounds for study were chosen so as to meet one or more of the following criteria: (1) compounds of some environmental concern, (2) compounds of similar structure such that extraction and analyses of several compounds could be accomplished simultaneously, and (3) compounds which would most likely (in our opinion) be completely mineralized if parent compound disappearance occurred and not simply transformed into some persistent product (we made no attempt to follow mineralization in 100% sludge since methane production from test compounds was an insignificant fraction of total methane production). The 18 compounds chosen were divided into two groups based on structure:

Group I	Group II
di-n-methylphthalate	o-methylphenol (o-cresol)
di-n-ethylphthalate	m-methylphenol (m-cresol)
di-n-butylphthalate	p-methylphenol (p-cresol)
butylbenzylphthalate	o-chlorophenol
di-(2-ethylhexyl)phthalate	m-chlorophenol
di-n-octylphthalate	p-chlorophenol
tri-m-cresylphosphate	m-methylbenzoate
	p-methylbenzoate
	o-chlorobenzoate
	m-chlorobenzoate
	p-chlorobenzoate

[1]Dr. Ami Horowitz, working in our laboratory, has investigated the degradation of aromatic compounds in anaerobic hypereutrophic lake sediments. Based on a comparison of 28 compounds incubated in both anaerobic lake sediment and 10% secondary sludge, there was approximately 65% agreement between the two habitats with regard to degradation or persistence.

For group I chemicals, 6 liters of anaerobic sludge were collected in 8-liter glass digestor bottles equipped with glass spigots for sampling, and stoppers with 1 psi check values for venting excess gas pressure. Phthalate esters plus tri-m-cresylphosphate were dissolved in acetone and added to digestor bottles in an amount to give 20 ppm of each compound. Digestor bottles were prepared in duplicate and incubated at 35°C for 8-10 weeks. One hundred milliliter samples were withdrawn routinely from the digestors, mixed with 10 ml toluene (to inhibit further biological activity) and dried for 17-23 hours at 50°C. Dried sludge was extracted in a Vir-Tis Omnimixer with two 150 ml aliquots of dichloromethane. The dichloromethane solution was centrifuged at 23,000 x g for 15 min to remove particulates and then filtered through a 0.2 um pore size Teflon filter. The filtered solution was concentrated by roto-evaporation to approximately 2-3 ml and applied to a florisil column (approximately 1 cm X 15 cm). The column was washed with 20 ml of hexane to elute extremely non-polar components, then with 20 ml of acetone. The phthalate esters plus tri-m-cresylphosphate were eluted in the acetone fraction. The acetone was evaporated to approximately 2 ml, internal standard added, and injected onto the GC column. A significant amount of colloidal material remained in the sample, but it was deposited onto the glass sleeve of the GC injector and did not interfere with the analyses; glass sleeves were changed periodically to avoid occlusion. Phthalate esters plus tri-m-cresylphosphate were quantified using a FID equipped GC with a 2 m 7% Dexsil glass column; the carrier gas flow rate was 35 ml/min N_2. Injector and manifold temperatures were 350° and 400°C respectively; the oven was temperature programmed from 150°-330°C at 12°/min. Phthalate esters plus tri-m-cresylphosphate concentrations were determined using known response factors to phenanthrene (internal standard). The limit of detectability was approximately 0.5 ppm.

Extraction efficiencies varied among compounds and among sludges (Figure 4). Recovery of di-n-methylphthalate and di-ethylphthalate was relatively poor. It appears that loss of these compounds was due both to volatilization during drying and loss during sample cleanup. Sludges also contained background peaks which co-chromatographed with certain phthalate esters. We suspect that some of these may be phthalate esters indegenous

in the sludge, however, we cannot be certain.

A total of three sludges were tested (Figures 5-7). Degradation is expressed as percent parent compound remaining with time; percentages have been corrected for extraction efficiencies and background peaks. Di-n-methyl-, di-n-ethyl-, di-n-butyl-, and butylbenzylphthalate were degraded in all sludges although the relative rates varied among compounds and among sludges. We cannot be certain whether or not di-(2-ethylhexyl)phthalate, di-n-octylphthalate, or tri-m-cresylphosphate were degraded (considering the accuracy and precision of extraction), however, concentrations in most sludges tended to decrease with time. Data for di-(2-ethylhexyl)phthalate were confounded due to the fact that a certain amount of leaching of this plasticizer from tygon tubing (attached to the spigot for sampling) undoubtedly occurred.[2] In general, relative rates of phthalate ester degradation, from fastest to slowest, were di-n-methylphthalate $\geq$ di-n-ethylphthalate > di-n-butylphthalate $\geq$ butylbenzyl phthalate.

A comparison of phthalate ester degradation, assessed by monitoring CH_4 production in 10% secondary sludge vs. parent compound disappearance in whole sludge revealed that degradation results were generally consistent, although not always comparable (Figure 8). Only di-n-methylphthalate in Adrian secondary sludge and di-n-butylphthalate in Jackson secondary sludge yielded comparable results in both test systems. Low methane recoveries (di-n-ethyl- and butylbenzylphthalate in Jackson sludge) or lack of methane production (di-n-ethyl- and butylbenzylphthalate in Adrian sludge) may, in large part be, attributable to lack of, or variability in, the viability of secondary sludge; separate phthalate esters were tested at different times using different batches of sludge.

In order to reassess the biodegradability of phthalate esters in 10% anaerobic sludge, separate experiments were initiated monitoring CH_4 production and substrate disappearance in 10% Jackson primary sludge. Serum bottles of 10% sludge were prepared and CH_4 production monitored as previously stated for the compound survey. Five milliliter samples of 10% sludge were periodically withdrawn from serum bottles and frozen until extracted. Individual replicate samples from bottles containing di-n-methyl-, di-n-ethyl-, and di-n-butylphthalate or butylbenzyl-,

[2]GC analysis of hexane extracts of tygon tubing indicated the presence of significant amounts of di-(2-ethylhexyl) phthalate.

di-(2-ethylhexyl)-, and di-n-octylphthalate were combined and extracted with 30 ml of hexane. After standing overnight to allow for phase separation, 25 ml of hexane (out of 30 ml) was withdrawn, evaporated to 2 ml and injected into a FID equipped GC with a 50 m SE-54 fused silica capillary column. Injection and manifold tempreatures were 300°C; initial oven temperature was 100°C for 5 min with a temperature program of 20°/min to 290°C; the carrier gas flow rate was 1 ml/min helium. Concentrations of phthalate esters were determined from standard curves. All incubations were run in triplicate.

Results for phthalate ester disappearance in 10% sludge were consistent with those using whole sludge (Figures 8 and 9), although the relative rates varied somewhat (Figure 9). Di-n-methylphthalate was degraded rapidly while di-n-ethyl-, di-n-butyl-, and butylbenzyl phthalate were all degraded at a slower rate. There was no evidence for the degradation of di-(2-ethylhexyl)- or di-n-octylphthalate. Methane production mirrored substrate disappearance (Figure 10) with percent theoretical CH_4 production ranging from 76%-103% (Figure 8). Based on CH_4 production and substrate disappearance data there does not appear to be a substantial lag time for phthalate ester degradation. With the exception of di-n-methyl- and di-n-ethylphthalate (40% and 30% loss respectively) there was no loss of extractable parent compound in autoclaved controls over the course of the incubation.

For group II chemicals, sludges were set up as previously stated, except that digestor bottles were fed 30 ppm of each compound. One hundred milliliter samples were routinely withdrawn and frozen until extracted. Fifty milliliter aliquots of sludge were adjusted to pH 2.0 and phenols and benzoates extracted with three 100-ml aliquots of dichloromethane. The dichloromethane solution was concentrated by roto-evaporation down to 3 ml and injected into GC for analysis of phenols. The dichloromethane solution was then taken to dryness, taken up in 5 ml of methanol and injected into HPLC for analysis of benzoates. Phenols were quantified using a FID equipped GC with 2 m Carbopak C-0.1% SP1000 glass column. Injector and manifold temperatures were 250°C, the oven was run isothermally at 205°C; the carrier gas flow rate was 20ml/min N_2. Benzoates were quantified using a HPLC with UV detector (set at 230 nm) and Partisil PXS 10/25 PAC column.

Samples were run at ambient temperature using a 95% methanol/5% 0.05 M Na acetate (pH = 4.5) buffer solvent system; the flow rate was 3.0 ml/min. Concentrations of compounds were determined from standard curves. Extraction efficiencies for substituted phenols were determined from 0 time samples (Figure 4).

A total of four sludges were tested. Data for Holt and Jackson sludges are shown in Figures 11 and 12; data for Mason and Ionia sludges were comparable. We were surprised by the degradation of o-chlorophenol in all four sludges since we had no prior evidence of its degradation. It is possible that o-chlorophenol may be transformed into some other persistent product, however it seems most likely to us that a dechlorination occurs leaving a readily degradable phenol moiety. We were disappointed in that compounds which should have been degraded — m-cresol and p-cresol — generally were not. Only in Mason sludge did p-cresol degradation occur, and then only after a 3 week lag. We are convinced that the sludges were viable, so presumably certain of the compounds which we added were inhibitory to degradation of m-cresol and p-cresol. We are suspicious of m-chlorophenol and p-chlorophenol. Both compounds were inhibitory to methane production in 10% secondary sludge at concentrations of 89 ppm (Figure 3). We have also observed that m-chlorophenol and p-chlorophenol were inhibitory to phenol degradation at 64 ppm in a phenol enrichment which we have maintained for 1 1/2 years. Data for methyl and chloro substituted benzoates are not shown because no degradation was observed for any of the compounds in any of the four sludges. Since we had prior evidence for degradation of m-chlorobenzoate in Holt sludge, degradation of this compound may also have been inhibited. However, at this time, we can make no general conclusions regarding the biodegradability of methylbenzoates or chlorobenzoates in anaerobic sewage sludge.

Comparison of degradation results among sludges (sludge survey)

Since potential users of this test method will presumably be obtaining anaerobic sludges from waste treatment plants in their own immediate area, it is important to have some understanding of how sludges vary with respect to their ability to degrade organic chemicals. In particular, we were interested in determining what types of compounds were consistently

degraded (such information would be extremely useful in selecting reference chemicals) and also attempting to evaluate the differences, if any, between "industrial" and "residential" sludges.

Bottles were set up as previously stated for the compound survey with the following exceptions: primary anaerobic sludge was used instead of secondary sludge and test bottles were run in triplicate. Twelve sludges were obtained from the mid-Michigan area. The sewage treatment plants from which these sludges were obtained vary considerably in size (inflow and number of people served) and percent industrial input (personal communication, John Phillips, former graduate, Pesticide Research Center, MSU) (Figure 13). Twelve compounds, all previously tested, were chosen to satisfy the following conditions: (1) diversity of compounds with respect to rate and extent of biodegradation, and (2) diversity of chemical structure and bond type. The compounds were:

polyethylene glycol-20,000	phthalic acid
2-octanol	di-n-butylphthalate
ethanol	di-n-octylphthalate
o-cresol	m-chlorobenzoic acid
m-cresol	propionanilide
p-cresol	chloroform

Degradation is expressed as percent theoretical methane production based on the stoichiometry of mineralization (see Appendix I). Lag time is the period of time (in weeks) required for approximately 20% of theoretical CH_4 production.

It is apparent that sludges varied in their ability to degrade different compounds in an 8-week incubation (Figure 14). All sludges were able to degrade ethanol, polyethylene glycol-20,000, p-cresol, and phthalic acid (with the exception of Charlotte), while m-cresol was degraded in at least six sludges. 2-Octanol, di-n-butylphthalate, and m-chlorobenzoate

proved more difficult to degrade; each compound was degraded by only 3-5 sludges. No sludge degraded all three compounds, although Jackson, Ann Arbor, and Adrian degraded two of the three. Propionanilide probably was partially degraded (in most sludges there was greater methane production in propionanilide bottles vs. control bottles); however, increased methane production was generally less than 20% theoretical, hence not significant enough to conclude degradation (we suspect that the propionic moiety is cleaved and degraded leaving a persistent aniline molecule). Percent degradation was generally greater than 70% for m-cresol, p-cresol, phthalic acid, and m-chlorobenzoate. Lower percent degradation for polyethylene glycol-20,000, 2-octanol, and di-n-butylphthalate may be, in part, due to their slower rates of degradation such that an 8 week incubation was not sufficient to allow for complete methane recovery. We are not certain why CH_4 recoveries from ethanol were low. It does appear that certain sludges were less active than usual, i.e. di-n-butylphthalate is normally completely degraded in 10% Jackson sludge with no apparent lag, yet in this study only 49% of theoretical CH_4 was recovered with a lag time of 5 weeks.

It is not obvious why sludges vary in their ability to degrade different compounds, other than the obvious yet unverifiable reason that different sludges have become acclimated to different chemicals due to prior exposure. With the possible exception of di-n-butylphthalate, there does not appear to be any apparent correlation between compound degradation and percent industrial input. With the exception of Pontiac, sludges with high input flows ($\geq 5 \cdot 10^6$ gal/day) degraded more compounds (seven), although Holt (flow = $1.1 \cdot 10^6$ gal/day) also degraded seven compounds. Based on these results, there does not appear to be any single criterion predictive of sludge versatility with respect to biodegradation.

Effect of length of storage on sludge viability

Ideally, fresh sludge inoculum should be obtained for use in all test incubations; however, depending on the distance between potential user and a suitable source of anaerobic sludge, this may be impractical. It is important then to know how long anaerobic sludge can be stored and under what conditions to maintain its viability.

Length of storage experiments were initiated using p-cresol, phthalic

acid (mono potassium phthalate), di-n-butylphthalate, and ethanol as test substrates with Jackson, Holt, Adrian, and Chelsea primary anaerobic sludges. Individual sludge samples were stored in tightly capped 200 ml bottles at 4°C until use, such that all incubations were begun with sludge from previously unopened bottles. Test bottles were set up as described in the sludge survey, except that $NaS \cdot 9H_2$ was not added to the medium. Gas production was monitored using a pressure transducer with multimeter and 3-way valve. The 3-way valve (with 25-gauge needle) was attached to the pressure transducer using stainless steel 1/16" tubing. At the beginning of all incubations, bottles were allowed to equilibrate at 35°C, for 2-4 hours, then vented to atmospheric pressure to equalize initial pressures. Gas pressure in bottles was measured and vented weekly. Experiments were begun weekly up to the 4th week of storage. Degradation is expressed as percent theoretical gas production based on the stoichiometry of CH_4 and CO_2 production and taking into account gas solubilities (see Appendix I).

Based on percent theoretical degradation (after an 8 week incubation), it is not apparent that a 4 week storage period had any detrimental effects on sludge viability (Figure 16). However, based on the lag time before significant gas production occurred, sludge viability was affected. This effect was particularly pronounced in Holt sludge (Figures 16-19). Lag times for degradation of p-cresol, phthalic acid and di-n-butylphthalate in 10% Holt sludge were significantly lengthened by storage of sludge beyond the second week, although rates of degradation after the lag were generally unaffected. It was also common for sludge stored for 3-4 weeks to show an initial net negative gas production before the onset of degradation. We are not certain as to the causes of this effect; we have no evidence for toxicity with any of these compounds. Ethanol degradation was unaffected by sludge storage with the exception of the fourth week where rate of degradation was somewhat slower. Jackson sludge was less affected by the 4 week storage (Figures 20-23). Lag times for degradation of p-cresol and phthalic acid were only slightly affected with rates of degration remaining approximately equal. Degradation of di-n-butylphthalate and ethanol were little affected, if at all. Again, in the case of phthalic acid, increased sludge storage resulted in initial net negative gas production. The effects of length of storage on lag times for all four sludges (weeks

required for 50% of net gas production) are summarized in Figures 24-26. Lag times for p-cresol degradation increased from 4 to 7 weeks for Holt sludge, 2-3 weeks for Jackson sludge, and 4-4.5 weeks for Adrian and Chelsea sludge. Lag times for phthalic acid degradation increased from 3 to 6 weeks for Holt sludge, 3 to 4 for Jackson sludge and 4 to 4.5 for Adrian and Chelsea sludges. Lag times for ethanol degradation were relatively constant. Based on these data, a 4 week storage period does not appear to be excessive for most sludges. We suspect that the viability of Holt sludge was adversely affected, primarily as a result of the long retention times (38-39 days) and low total solids (approximately 1.7%) of the primary sludge; in essence, the organisms were starved for carbon. We feel that the use of fresh sludge is always preferrable, if it can be conveniently obtained.

Effect of mineral salts media on gas production from 10% sludge

Since the purpose of the test method is to assess anaerobic biodegradation potential it is desirable that the rate and extent of xenobiotic degradation be maximized. In order to maximize degradation it is necessary that the nutritional requirements for each component of the methanogenic consortium be supplied. Presumably, in whole sludge this is not a problem, however, in 10% sludge it is possible that some critical mineral nutrient may be diluted below its optimum concentration. We chose to attack this question from three directions: (1) review the literature on methanogensis in search of studies demonstrating nutritional requirements, (2) survey the anaerobic literature for content and concentration of mineral salts and metals commonly found in anaerobic media and compare these with minerals and metals found in 10% sludge, and (3) based on 1 and 2, compare experimentally those media most likely to be proposed for the test method.

We are aware of few studies where the mineral nutritional needs of methanogens or methanogenic consortia have been extensively investigated (most studies have concentrated on organic nutritional requirements). Taylor and Pirt (21) reported that growth of Methanobacterium thermoautotrophicum (a lithotrophic methanogen) was enhanced by 11 mM NH_4^+ and 0.05 mM Fe^{3+} as compared to concentrations five times lower. Patel, et

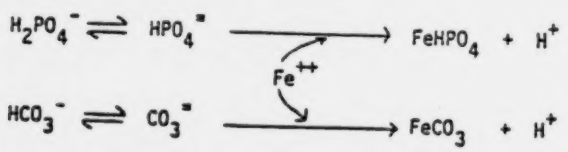
al. (17) reported that growth of Methanobacterium strain M.O.H., Methanospirillum hungatii GPI, and an unidentified strain similar to Methanobacterium formicicum (all lithotrophic methanogens) was optimal at iron concentrations in the range of 0.3-0.9 mM. Khan, et al. (12) reported that maximum rates of methane production from cellulose by a methanogenic consortium were achieved at HCO_3^- and Fe^{3+} concentrations of 16-24 mM and 0.4-0.6 mM respectively, while nitrogen concentrations ranging from 3.5-30.0 mM and phosphate from 1.5-12.0 mM had little or no effect on cellulose degradation. Hoban and Berg (9) reported that iron additions of 5-20 mM resulted in stimulation of methane production from acetate up to 100% in cultures enriched for heterotrophic methanogens. Such large additions were necessary in order to increase the concentration of soluble iron; iron was precipitated in the form of FeS, $FeHPO_4$ and $FeCO_3$. We were particularly interested in this observation since carbon flow through acetate is of key importance in methanogenic consortia. We repeated the work of Hoban and Berg using 10% sludge and our enrichment medium to determine if increased iron additions would result in an increased rate of methanogenses.[3]

Test bottles were set up as previously described for the sludge survey, except that a 3 mM phosphate buffer was used. Adrian (no substrate added) and Jackson (50 ppm carbon diethylene glycol added) 10% sludge inocula were prepared, with addition of 0, 5, 10 or 20 mM $FeCl_2 \cdot 4H_2O$. Concentrations of iron, phosphorous, and other minerals in the 10% sludge solution were measured at the start of the incubation. Two milliliters of sludge solution were removed from each bottle, filtered through a 0.45 um pore size Millipore filter, acidified with HCl, and stored at room temperature until analyzed for metal content on a plasma emission spectrometer. Methane production was monitored weekly using a FID equipped GC. At the termination of the Adrian experiment, the pH of bottles was recorded.

Methane production in both Adrian and Jackson sludge was inhibited by addition of iron to the medium (Figures 27-28). The level of inhibition appeared to be directly correlated with the amount of iron added. With higher iron additions, levels of soluble iron also increased but the majority of iron was precipitated out in the form of $FePO_4$ (soluble PO_4 was

[3]These experiments were performed primarily by Susan Frasier, under the direction of Dr. Tiedje for the undergraduate course MPH 490: Special Problems.

almost completely absent at 20 mM Fe), and probably as $FeCO_3$ as well (Figure 29). The pH of medium receiving 20 mM iron was depressed (6.1-6.2) well below the optimum for methanogenesis which probably caused the inhibition of methane production. Apparently, our buffering capacity (3 mM PO_4 and 10% CO_2) was insufficient to offset the effect of the iron addition. This can be visualized from the following scheme (assuming $FeHPO_4$ and $FeCO_3$ are the primary precipitates)



This is probably oversimplified, however, it illustrates how the buffer capacity could have been exceeded. Hoban and Berg used a 40% CO_2/60% N_2 gas mixture such that their total bicarbonate buffer was approximately 56 mM, sufficient to buffer the pH. Based on results with our present medium, we feel that the addition of high levels of iron is not advisable.

A survey of the anaerobic literature reveals that the concentrations of minerals in media routinely used for culturing methanogens and methanogenic consortia varies from one to two orders of magnitude (Figure 30) (4,12,14,17,18,21,22,25,26). This is probably indicative of the fact that the concentration of most minerals and metals is, within reason, not particularly critical. Sommers (19) conducted a survey of sewage sludges from New Hampshire, New Jersey, Indiana, Illinois, Michigan, Wisconsin, Minnesota and Ohio in which he reported the range, median, and mean concentration for several elements (Figure 30). A comparison of anaerobic media with 10% sludge indicates that, with the exception of perhaps K, NH_4+ and Co, all elements are present in 10% sludge in ample supply.

Experiments were initiated with Jackson and Holt sludges to directly compare the ASTM medium, our enrichment medium, and phosphate buffer alone. Bottles were set up as previously described for length of storage except that 0.5 mM sulphide was included in our enrichment medium; phosphate

buffer medium contained no added sulphide (Figure 30). Gas production was monitored using a pressure transducer with multimeter and 3-way valve (a 20-guage needle was used in place of a 25-guage needle). Unfortunately, it was necessary to terminate this experiment after 6 weeks of incubtion due to extreme variability and anamolous data from all three media. New experiments were initiated with Jackson, Holt, Mason and Ionia sludges to compare the ASTM medium, our enrichment medium (O.E.M.) and a supplemental medium (S.M.) consisting of 3 mM $PO_4^=$ buffer, 9 mM K, 10 mM NH_4^+, and 10 uM Co (those elements most likely to be in low concentrations in 10% sludge). Mineral and metal composition of 10% Jackson, Holt, Ionia and Mason sludges are listed in Figure 31. One gram samples of dried sludge were digested sequentially with liberal amounts (20-30 ml) of HNO_3, HF and $HClO_3$ in 50 ml Teflon beakers at 110°-130°C. The remaining metals and minerals were dissolved in 6N HNO_3 with 1000 ppm LiCl and analyzed on a Plasma Emission Spectrometer. Substrates tested were p-cresol (introduced directly into serum bottles with a 10 ul microsyringe), phthalic acid (introduced into serum bottles in 0.5 ml water as monopotassium phthalate), di-n-butylphthalate (introduced directly into serum bottles with a 10 ul syringe) and m-chlorobenzoic acid (introduced into serum bottles in 0.5 ml di-ethylether and the ether allowed to evaporate for $\geq$ 2 hours). Medium performance was judged based on three criteria: (1) maximum background gas production (we assume that any inhibition of gas production is potentially deleterious to biodegradation), (2) maximum gas production from test compounds, and (3) minimum lag times.

In the aborted medium experiment (6 week incubation) we observed that gas production was markedly inhibited in control bottles containing the ASTM medium relative to our enrichment medium, and in both media relative to phosphate buffer alone (Figure 32). This was not unexpected for the ASTM medium, because the CO_2 gas phase and $NaHCO_3$ called for in the medium are not in equilibrium (see Appendix I). Although we stir our 10% sludge solution before dispensing bottles, this agitation is neither sufficient nor prolonged enough to allow for $CO_2(g)$ to establish equilibrium with $CO_2(aq)$ and HCO_3^-; the rate of phase transfer is too slow. When bottles containing the ASTM medium were measured prior to venting, after equilibration at 35°C, they consistently pulled a vacuum; bottles

containing our enrichment medium or phosphate buffer consistently had overpressures of 1-3 ml. In the completed medium experiment, we again observed an inhibition of gas production in control bottles containing the ASTM medium relative to our medium, even though a sufficient amount of $NaHCO_3$ had been added to bring aqueous and gas phases into equilibrium (3.6 g $NaHCO_3$/liter). We also observed a slight inhibition of gas production in control bottles containing our enrichment medium relative to the supplemental medium. In an attempt to understand this phenomenon, 100 ml aliquots of 10% sludge were dispensed sequentially from the same batch of 10% sludge (10% CO_2/90% N_2 headspace) containing phosphate buffer alone, phosphate buffer + our mineral salts, and phosphate buffer + our mineral salts + 0.5 mM sulphide, or (separate batch of 10% sludge) no addition, ASTM mineral salts, ASTM mineral salts + 2 mM sulphide. We found that there was no effect of mineral salts additions on gas production after a 2 week incubation (Figure 32); however, addition of both 0.5 mM and 2 mM sulphide resulted in inhibition of gas production with the level of inhibition inversely proportional to sulphide concentration. We are not certain if this is primarily an effect on the CO_2(g) bicarbonate equilibrium (sodium sulphide solutions are extermely alkaline) or on the biology. Sulphide is known to inhibit methanogenesis at higher concentrations, however 2 mM is generally considered to be a safe concentration.

Percent theoretical gas production for substrates incubated in serum bottles containing the ASTM medium was uniformly higher in all four sludges than with either our enrichment medium (O.E.M.) or the supplemental medium (S.M.) (Figure 33). This effect was particulrly pronounced for phthalic acid and m-chlorobenzoic acid. Since the same stock solutions of phthalic acid and m-chlorobenzoic acid were used for amending bottles receiving the three different media, it is highly unlikely that ASTM medium bottles were consistently overfed. It is more likely that the over production of gas was attributable to abiological rather than biological causes. This is particularly true in the case of apparent gas production from m-chlorobenzoic acid in sludges for which we had no prior evidence of degradation. Both phthalic acid and m-chlorobenzoic acid were amended into serum bottles as mono-acids (the second carboxyl group of monopotassium phthalate has a pKa of 4.75 while m-chlorobenzoic acid has a pKa of 4.5)

such that at a pH of 7.0, both compounds would be expected to be deprotonated. Since the ASTM medium's sole buffer system consists of 30% $CO_2(g)$ - HCO_3^-, any introduction of protons would result in a flux of CO_2 gas into the headspace ($HCO_3^- + H^+ \rightleftharpoons H_2CO_3 \rightleftharpoons H_2O + CO_2$). Our enrichment medium consists of a 2 mM $PO_4^=$ system in conjunction with 10% $CO_2(g)$-HCO_3^-, while the supplemental medium consists of 3 mM $PO_4^=$ in conjunction with 10% $CO_2(g)$ - HCO_3^-. The phosphate ions would form an alternate (or competing) buffer system, such that fluxes of CO_2 in the headspace would be diminished. That this may be the case is supported by the fact that much or all of the difference in gas production between the ASTM medium and our enrichment medium in serum bottles containing phthalic acid or m-chlorobenzoic acid with 10% Holt or 10% Ionia sludge can be accounted for in the first 1-2 weeks of incubation (Figures 35,37,39,and 41). Based on past experience, this is considerably shorter than the expected lag period. With the exceptions of p-cresol and di-n-butylphthalate in 10% Holt sludge (Figures 34 and 36), there does not appear to be any significant effects of different mineral salts media on lag times or rates of degradation (Figures 35 and 37-41). Results for Jackson and Mason 10% sludges are consistent with those for Ionia 10% sludge.

We feel that the most important components of the medium are the buffer system and the reductant. Based on these experiments, we feel that a 2 or 3 mM phosphate buffer system is advisable in conjunction with a 10% $CO_2(g)$ - HCO_3^- system. We do not feel that the addition of sulphide is necessary, however, if added, we recommend that the concentration not exceed 1 mM. Iron concentrations greater than 2 mM are also not recommended. We have obtained satisfactory results with our enrichment medium; however, any standard anaerobic mineral salts medium would probably be acceptable.

Effects of O_2 on gas production in 10% sludge

Since the purpose of this test method is to assess anaerobic biodegradation, it is important that O_2 be excluded from test bottles. In the case of accidental O_2 intrusion, however, it is important to have some means of detecting the extent of O_2 contamination in bottles so that they can be ignored or discarded. Almost all anaerobic media contain resazurin

as a redox indicator (resazurin is colorless at Eh < -0.100 mv and pink when oxidized). Although resazurin is a very sensitive indicator of O_2 intrusion in anaerobic media, we were not aware of any information indicating the sensitivity of resazurin to O_2 intrusion in 10% sludge. Based on experience with injecting O_2 into bottles with 10% sludge, we have found that a minimum of 5% (generally 5%-10%) O_2 in the headspace is required for a pinkish color to appear in the water column, with or without sulphide. A 5% O_2 atmosphere corresponds to a headspace composed of 25% air. Using even poor anaerobic technique, it is unlikely that O_2 contaminated bottles would be detected by this indicator.

We were interested in the capacity of 10% sludge to consume O_2 and whether slight O_2 additions might effect gas production. Bottles were set up as previously stated in the compound survey, except that 70 ml bottles were filled with 40 ml of 10% Adrian sludge and 0-5 ml of O_2 injected into the headspace. Oxygen concentrations were quantified daily using a Carle GC equipped with 2 m Porapak-Q column and microthermister detector; the carrier gas flow rate was 40 ml/min He. All bottles were maintained static. Oxygen was consumed at a rate of approximately 1 ml per day (Figure 42). Bottles containing 3, 4 or 5 ml of O_2 had a pink gradient at the top of the water column at zero time. After 24 hours a pinkish color was visible in bottles having received 4 and 5 ml of O_2 and after 48 hours the water column in all bottles was colorless.

In a separate experiment bottles with 10% Jackson sludge were set up as previously stated for length of storage experiments. After equilibrium at 35°C and venting of bottles, 0, 1, 2, 3 or 4 ml of gas was removed from the headspace and replaced with an equal amount of O_2. Half of the bottles were incubated with benzoate as substrate. After 16 hours, bottles receiving 4 ml of O_2 (6.7% O_2 in the headspace) were slightly pink at the water-gas interface, however, if the bottles were even slightly agitated the pink disappeared. No pink was obvious after 40 hours. Oxygen addition affected gas production data significantly (Figure 43). Gas production after one week decreased proportionally to increasing O_2 additions. This was expected since the O_2 would have been consumed leaving a partial vacuum. A decrease in gas production was still evident after two weeks and was particularly pronounced in bottles receiving benzoate as substrate.

Presumably, this was due to inhibition of methanogenesis and loss of benzoate carbon to aerobic metabolism. Although it is clear that significant variability can be introduced as a result of O_2 intrusion in the absence of its detection, we do not feel that this presents a serious problem if careful anaerobic technique is observed.

Effect of chemical concentration on gas production in 10% sludge

An objective of this research was to optimize the conditions under which anaerobic biodegradation can occur, yet retain a relatively simple assay for biodegradation which can be performed quickly and inexpensively. The only sure way to maximize biodegradation potential is to work with fresh whole sludge, while the most simple and inexpensive procedure for assessing biodegradation is to monitor gas production. Unfortunately, the two are incompatible. Total gas production from whole sludge is much too great to allow for quantitation of small amounts of gas from xenobiotic mineralization. Conversely, in order to decrease background gas production, it is necessary to age or dilute whole sludge; both result in a decrease or dilution of the biological activity. The goal, then is to find some suitable compromise between maximizing viability and diversity, hence, maximizing chances for biodegradation to occur, and minimizing background gas production such that gas production from xenobiotics can be accurately quantified. The crucial question in determining this compromise is, what concentration of xenobiotic is most appropriate for such a screening method. If low concentrations of xenobiotics are to be tested then steps must be taken to decrease background gas production, while if higher concentrations are acceptable less dilution is necessary and desirable to minimize potential toxic effects. The ASTM method proposes that compounds be tested at a concentration of 50 ppm carbon, however, we were not aware of any experimental justification for this recommendation.

We initiated experiments with 10% Jackson sludge to investigate the possibility of using lower concentrations of substrate, and to investigate the potential toxic effects of phenol, p-cresol, benzoate, and phthalate at higher concentrations (benzoate and phthalate were added in the deprotonated form). Bottles were set up as previously described for the length of storage experiment. Substrates were tested at 25 ppm, 50 ppm, 100 ppm, and 200 ppm carbon.

Percent theoretical gas production was not appreciably affected by initial substrate concentration; apparently toxicity was not a problem (Figure 44). Standard deviations were greatest for low substrate concentrations and tended to decrease with increasing substrate concentrations. Based on these results, it is possible that substrates could be tested at 25 ppm carbon; however, this concentration is near the limits of resolution for the test. If lower concentrations were recommended it would be necessary to pre-age the sludge before use to reduce background gas production. This could be accomplished by incubating whole sludge at 35°C for approximately 5 days prior to use. Within this period of time, the majority of gas production should occur (personal communication, John Eastman, Assistant Professor, Department of Civil and Sanitary Engineering, MSU). If a concentration of 50 ppm carbon is acceptable, however, we do not feel that a pretreatment is necessary. Test compound concentrations of greater than 50 ppm carbon may also be acceptable, however, they do not appear necessary.

Reproducibility of Results

One of the most important aspects of any test method is the ability to obtain reproducible results. Observations which cannot be repeated are of little interpretive value and represent a waste of time and resources. We have evaluated the variability associated with the analytical aspects of the method, the variability among replicate determinations using the same batch of sludge, and the variability in the final interpretation among different batches of sludge.

We have found the pressure transducer to be a reliable instrument. Gas pressure measurements can be made with extreme accuracy and precision (Figure 45). The standard curve shown in Figure 45 is the average of 13 different determinations over an 8 week period. As illustrated by Figure 45, there is no significant variability associated with the analytical method. We have had, from time to time, difficulty with variability among replicates, signified by high standard deviations (Figure 15). During the length of storage experiments we observed that water vapor was condensing in the 25-gauge needle and 3-way valve such that needle and valve had to be flushed with air periodically to avoid occlusion. For the effect of

mineral salts medium experiments, a 20-gauge needle was used. Either as a result of this switch or closer attention to detail, standard deviations for the effect of mineral salt medium experiments were somewhat improved, although there was still occasional instances of high variability among replicates. The source of this variability is not clear. It does not appear to be associated with substrate degradation; standard deviations (average for all four sludges in ml of gas) for separate substrates were control (0.56), p-cresol (0.64), phthalic acid (0.72), di-n-butylphthalate (0.52) and m-chlorobenzoic acid (0.46). Neither does the variability appear to be associated exclusively with gas pressure measurements as opposed to methane measurements; the standard deviation for methane production in control bottles (average of 12 sludges in sludge survey) was 0.38 ml of CH_4. We suspect that the greater part of this variability is due to lack of complete homogeneity in the 10% sludge. Although we vigorously stir the 10% sludge suspension while dispensing 100 ml aliquots into serum bottles, small discrepencies in the distribution of degradable organic matter would be sufficient to cause variability in gas production.

Reproducibility of results using the same batch of sludge does not appear to be a problem. With the exception of a few anomalous results in the length of storge experiments, no sludge failed to degrade or not to degrade test compounds when incubated using the same batch of sludge. Even more importantly, there was reproducibility between batches of the same sludge, collected over a 1 1/2 year span. When degradation results for several substrates used as test compounds in as many as five separate experiments are compared (compound survey, sludge survey, effect of length of storage, effect of mineral salts media, and effect of substrate concentration), there are only four discrepancies out of a possible 66 comparisons (Figure 46). This does not include the data from the aborted effect of mineral salts media experiment.

We feel that with careful technique and a minimal knowledge of anaerobic sewage sludge, this method should yield relatively consistent and reproducible results. We do not believe there should be any difficulty with making reliable conclusions using this method.

Discussion of Significant Test Parameters and the Rationale for Our Recommendations

What should be the ratio of inoculum to test compound concentrations?

We have routinely used a 10% sludge inoculum with 50 ppm carbon test compound in our studies, and see no reason to recommend other conditions. Based on our experience, a 10% sludge inoculum will, in general, yield 10-40 ml of gas, depending on the retention time and total solids of the sludge. A 50 ppm carbon addition will yield between 5.5 to 8.5 total ml of gas, depending on the stoichiometry of mineralization. Even though the background gas production may be significantly higher than the gas production from the test compound, we feel that the accuracy and precision of the test are sufficient to allow for assessment of biodegradation. As stated previously, we feel that 50 ppm carbon substrate is the lowest reasonable concentration which yields sufficient gas production over controls to be quantifiable. Concentrations greater than 50 ppm carbon are more easily quantifiable, however, they are not necessary and may lead to toxicity. It is quite possible that some compounds which are relatively slowly degraded in whole sludge may not be appreciably degraded in 10% sludge; however, certain compromises are necessary in order to decrease background gas production.

What should be the source of inoculum?

We have had only limited experience with anaerobic lake sediments but have found them more difficult to work with in this test system. Also, based on work by Dr. Horowitz in our laboratory, lag times in sediment are generally longer than in sludge (10). Primary anaerobic sludge with a retention time of 15-30 days is most desirable. Shorter retention times may result in high background gas production, while longer retention times may result in attenuation of sludge viability, particularly during storage. Although total solids and volatile solids measurements are not always indicative of background gas production, in general, we have found that sludges with a percent organic matter (total solids X volatile solids) of approximately 1.0%-2.0% provide satisfactory results. We strongly recommend that potential users find a waste treatment plant where anaerobic

digestors have been operated successfully in the past and are likely to be so in the future. Although this is no guarantee that different batches of the same sludge will always perform identically, results are more likely to be consistent over time. At present we have insufficient data to allow us to predict which types of anaerobic sludges are most likely to degrade the widest range of xenobiotics.

We have not experimented with the mixing of different sludges together (or sludge with sediment and/or soil) in an attempt to increase the xenobiotic degrading versatility of the anaerobic inoculum. However, we feel that this approach is promising. Since sludges do vary with respect to their xenobiotic degrading capacity, a combination of the most active sludges should, ideally, result in a maximizing of the biodegradation potential.

How should the inoculum be stored or pretreated?

Based on our results discussed previously, we feel that most sludges can be stored for up to 4 weeks at 4°C (in tightly capped containers) if necessary; however we recommend that fresh sludge be obtained whenever possible. It is clear, based on lag times for substrate degradation, that storage can have a detrimental effect on sludge activity, particularly for those sludges with long retention times and low organic matter contents. If, however, sludges with retention times of less than 30 days and percent organic matter of greater than 1.0% are used, attenuation in activity is not likely to be a serious problem. Based on our experience working with a 10% sludge inoculum, we do not feel that a pretreatment of the sludge is warranted.

What should be the composition of the test medium?

Based on a survey of the literature and our own experience, we do not feel that there is a strong basis for preferring any particular anaerobic medium. In most cases, 10% sludge alone is likely to contain all mineral and metal nutrients in ample supply, with the possible exceptions of K, NH_4^+, and Co. However, inclusion of a complete mineral salts medium is probably advisable as insurance. We recommend the use of a 2 or 3 mM phosphate buffer system in conjunction with 10% $CO_{2(g)}$ - HCO_3^-. We do not

feel that the addition of sulphide is necessary; however, if used, concentrations should not exceed 1 mM.

What should be the length of incubation?

We feel that an 8 week incubation is advisable. Based on our own work and that of Healy and Young, a 4 week incubation is simply not sufficient to allow for biodegradation of more persistent chemicals. Longer incubations are probably not justifiable. Ten percent sludge, like whole sludge, has a finite shelf life beyond which components of the methanogenic consortium cannot be expected to remain viable. Although we have not performed experiments designed specifically to answer this question, we have observed that background gas production in all control bottles has ceased by the 8th week. We recommend that compounds be incubated for 8 weeks or until biodegradation is complete, defined as two consecutive weeks without net gas production. If gas production is still in progress at the 8th week, incubations should be continued until gas production has ceased. We recommend that incubations be conducted at 35°C, since most primary anaerobic digestors are operated in this temperature range.

How should test substrates be introduced into sludge bottles?

The ASTM method proposes that bottles be incubated overnight before testing begins to assure anaerobiosis, and that water insoluble materials first be dissolved into dioxane, then introduced into 10% sludge bottles. Based on our O_2 intrusion experiments, we do not feel that it is necessary to hold bottles overnight to check for O_2 contamination because the redox indicator (resazurin) is not sensitive to low levels of O_2 contamination. With proper anaerobic technique, O_2 instrusion should not pose a serious problem.

We have not extensively investigated the effect of potential organic diluents on methane and/or gas production in 10% sludge. Based on a limited survey of common organic solvents incubated in 10% sludge (amended with 1 mM glucose) at a concentration of 0.1%, we found four solvents (acetonitrile, aniline, dioxane and pyridine) which were neither inhibitory to methanogenesis nor degraded (Figure 47). Aside from the fact that some of these compounds have undesirable characteristics i.e., dioxane is an

animal carcinogen, it is not possible to definitely assert that the introduction of these solvents into 10% sludge at concentrations probably approaching 0.1% would have no effect on the fermentative populations of the methanogenic consortium. We prefer to first dissolve water insoluble solids into di-ethylether, dispense the ether solution into serum bottles, and allow the ether to evaporate for $\geq$ 2 hours prior to filling with 10% sludge. We have found no residual effects of the ether after a 2 hour evaporation period. Liquids can be dispensed directly with a microsyringe while water soluble solids should be dissolved in water, then dispensed into bottles. Unfortunately, insoluble polymers must be weighed out individually.

How should the volume of gas be measured?

We have had considerable experience in monitoring both CH_4 production (via gas chromatography) and gas production (via pressure transducer) as a means of assessing biodegradation, and feel that there are advantages and disadvantages to both methods. Pressure transducer measurements are extremely accurate and precise, easily and rapidly obtained, and the required instrumentation (pressure transducer, multi-meter, 3-way valve, and 1/16" tubing) relatively inexpensive. Unfortunately, accurate measurement of biological CO_2 and CH_4 production is complicated by the fact that CO_2 is highly soluble in and reacts with water, such that small variations in conditions affecting CO_2 solubilities can result in errors in accurately assessing the extent of biodegradation. Gas chromatography allows for accurate and sensitive quantitation of methane gas. Also, since methane is produced solely as a result of biological activity and is relatively insoluble in and does not react with water, measurements of methane production are less sensitive to abiological fluctuations. Monitoring of methane production also appears to be a more sensitive indicator of inhibition due to toxicity. Based on our limited experience, compounds which cause complete inhibition of methane production (chloroform) show moderate levels of gas production, although significantly less than controls. This could be due to CO_2 flux into the headspace as a result of acid production by fermenters or simply a result of volatility of the compound itself. However, measuring methane via gas chromatography is

relatively slow, and requires moderately sophisticated instrumentation (a gas chromatograph and accessories). Considering the speed, accuracy, precision and cost effectiveness with which gas pressure measurements can be made, we feel that the use of pressure transducer and multimeter is the method of choice, particularly for a general screening method such as this. We have routinely corrected for gas solubilities when calculating percent theoretical degradation though this is not necessary in making a decision as to whether compound degradation has occurred (See Appendix I).

Gas pressure measurements were made by inserting a 20-guage needle, attached to a Hamilton Miniature Inert 3-way valve, into serum bottles. The 3-way valve was attached to a Unimeasure pressure transducer (Grants Pass, Oregon) equipped with a P-8 adapter (8 psi maximum response) via 10 cm of 1/16 in stainless steel tubing (fitted to pressure transducer) and a 16-guage luer needle (fitted to 3-way valve) connected by a 1/16" in Swagelok union. The signal from the transducer was quantified using a Fluke multimeter (Mountlahe Terrace, Washington). Serum bottles were shaken vigorously before pressure measurements were taken, and excess gas vented after measurements were taken to avoid cumulative gas pressures beyond the response range of the P-8 adapter.

What should the reference chemical(s) be?

The purpose of incubating sludges with reference chemicals is to ascertain that the sludges are biologically active and to characterize the sludges with respect to their overall versatility. Any readily degradable substrate such as ethanol can be used to quickly determine whether or not a sludge is active. However, a more rigorous test of activity and viability may be desirable. We have used p-cresol and phthalic acid throughout most of our testing. We feel that one or both of these compounds may be useful as reference chemicals because (1) both compounds appear to be uniformly degraded in anaerobic sewage sludge (sludge survey), (2) degradation results for both compounds have been consistently reproducible (reducibility of results), and (3) lag times before degradation have varied from 2 to 6 weeks depending on the age and source of sludge. We do not feel that we have sufficient knowledge or experience to recommend a group of chemicals which could be used to characterize sludges with respect to

their biodegradational versatility. Although it is clear that sludges vary with respect to their ability to degrade specific compounds, it is not apparent that this is predictive of their ability to degrade general classes of compounds of like structure.

Appendix I

Calculation of percent theoretical degradation

In order to ascertain CH_4 and CO_2 production from a particular substrate, the stoichiometry of mineralization must be determined. This can be done as proposed in the ASTM method. Using benzoic acid as an example:

$$C_7H_6O_2 + 12\ H_2O \rightarrow 7CO_2 + 15H_2$$
$$7CO_2 + 15H_2 \rightarrow 3.75\ CH_4 + 3.25\ CO_2 + 7.5\ H_2O$$

$$C_7H_6O_2 + 4.5\ H_2O \rightarrow 3.75\ CH_4 + 3.25\ CO_2$$

At a concentration of 50 ppm carbon in a 100 ml aqueous phase and 35°C, the volume of gas produced is:

5 mg carbon benzoic acid x 5/0.6885 = 7.27 mg benzoic acid
(0.6885 = fraction of molecular weight due to carbon)

7.27 mg benzoic acid = 0.0595 m moles benzoic acid/100 ml

x 3.75 ; x 3.25

0.2232 m moles CH_4 + 0.1934 m moles CO_2 = 0.4167 m moles total gas

0.4167 m moles gas at 35°C = 10.5 ml of gas

Since all substrates are provided at 50 ppm carbon, the theoretical gas yield for all substrates, regardless of structure, is 10.5 ml. If methane production is monitored (as opposed to total gas) the stoichiometry of substrate conversion to CH_4 must be determined. Examples of determinations involving different structural groups are given below.

o-, m-, p- aminobenzoic acid

$$C_7H_7O_2N + 12H_2O \rightarrow 7CO_2 + 14H_2 + NH_3$$
$$7CO_2 + 14H_2 \rightarrow 3.5\ CH_4 + 3.5\ CO_2 + 7H_2O$$

$$C_7H_7O_2N + 5H_2O \rightarrow 3.5\ CH_4 + 3.5\ CO_2 + NH_3$$

o-, m-, p-chlorobenzoic acid

$$C_7H_5O_2Cl + 12H_2 \longrightarrow 7CO_2 + 14H_2 + HCl$$

$$7CO_2 + 14H_2 \longrightarrow 3.5\ CH_4 + 3.5\ CO_2 + 7H_2O$$

$$C_7H_5O_2Cl + 5H_2O \longrightarrow 3.5\ CH_4 + 3.5\ CO_2 + HCl$$

tricresylphosphate

$$C_{21}H_{21}O_4P + 42\ H_2O \longrightarrow 21CO_2 + 51\ H_2 + H_3PO_4$$

$$21\ CO_2 + 51\ H_2 \longrightarrow 12.75\ CH_4 + 8.25\ CO_2 + 25.5\ H_2O$$

$$C_{21}H_{21}O_4P + 16.5\ H_2O \longrightarrow 12.75\ CH_4 + 8.25\ CO_2 + H_3PO_4$$

parathion

$$C_{10}H_{14}O_5NPS + 15H_2O \longrightarrow 10\ CO_2 + 18\ H_2 + NH_3 + H_3PO_4 + H_2S$$

$$10\ CO_2 + 18\ H_2 \longrightarrow 4.50\ CH_4 + 5.50\ CO_2 + 9.0\ H_2O$$

$$C_{10}H_{14}O_5NPS + 6H_2O \longrightarrow 4.5\ CH_4 + 5.5\ CO_2 + NH_3 + H_3PO_4 + H_2S$$

As calculated above, 50 ppm carbon of any chemical yields a total of 10.5 ml gas (CH_4 + CO_2). However, since both CH_4 and CO_2 are soluble in water, one does not actually measure 10.5 ml of gas (assuming 100% mineralization) in the headspace. The quantity of methane dissolved in the aqueous phase is a function of temperature and can be calculated according to the equation:

$$Vg = \frac{V_t}{1 + \frac{L \cdot A}{G}}$$

where Vg = volume of gas in headspace
V_t = total volume of gas in bottle
A = volume of aqueous headspace
G = volume of headspace
L = Ostwald coefficient[1] (defined as mls gas dissolved in 1 ml of water at 1 atm partial pressure of gas)

Assuming a 100 ml aqueous phase, 60 ml gas phase, and 35°C (L = 0.0296), if V_t is arbitrarily set to 100 Vg = 95.3; or 95.3% of total CH_4 in the bottle will be in the headspace.

Unfortunately, the loss of CO_2 to the aqueous phase is considerably greater. Not only is CO_2 highly soluble in water (L = 0.666 at 35°C), it also reacts chemically with water to form carbonates according to the following scheme:

$CO_{2(g)}$ gas phase

$$CO_{2(aq)} + H_2O \rightleftharpoons H_2CO_3 \overset{+H}{\rightleftharpoons} HCO_3^- \overset{+H}{\rightleftharpoons} CO_3^{=} \text{ (dissolved) liquid phase}$$

$CO_3^{=}$ (precipitate) solid phase

The quantity of CO_2 dissolved in the aqueous phase is a function of both pH and temperature. At a given pH, one can calculate the fraction of dissolved carbonate present as $CO_{2(aq)}$ according to the equation (from Aquatic Chemistry, Stumm and Morgan, Wiley-Interscience, 1970, p 120):

---

[1]Although the Bunsen coefficient (α) is generally more familiar and widely used than the Ostwald coefficint (L), we chose to use the Oswald coefficient because it gives the volume of dissolved gas at temperature (35°C), whereas the Bunsen coefficient gives the volume of gas assuming 0°C, regardless of the actual temperature. Since theoretical gas production is calculated at 35°C, the Ostwald coefficient is more accurate. The two can be equated through the expression

$$L = \frac{T \cdot \alpha}{273.15}$$

$$\alpha_o = (1 + \frac{K_1}{[H^+]} + \frac{K_1 \cdot K_2}{[H^+]^2})^{-1}$$

where $pK_1 = 6.3$, $pK_2 = 10.25$

Then, using the equation:

$$Vg = \frac{V_t}{1 + \frac{L \cdot A}{\alpha_o \cdot G}}$$

the percent of total inorganic carbon (excluding precipitates) present as CO_2 in the headspace at a given pH and temperature, can be calculated. The effect of pH on CO_2 solubilities is illustrated by the following calculations:

pH	α_o	Percent of CO_2 in headspace at 35°C
7.2	0.117	9.1
7.1	0.1367	11.0
7.0	0.1663 (0.1900[a])	13.0 (14.6[a])
6.9	0.2007	15.3
6.8	0.2402	17.8

a = adjusted for approximate ionic strength

Assuming a pH of 7, and 10% CO_2 headspace in equilibrium with the aqueous bicarbonate pool, one can calculate that there should be a total of 41.1 mls of gas in the bottle. We add 1.2 g $NaHCO_3$/liter medium which would give a total of 42.1 mls of gas in the bottle. We have found that after dispensing medium into bottles and allowing for equilibration at 35°C, there are 1-3 ml of gas above atmospheric pressure in the headspace.

We have determined empirically that approximately 37% of $CO_2(g)$ added to the headspace of serum bottles containing 10% sludge (to simulate biological CO_2 production) remains in the headspace; the rest is dissolved in the aqueous phase. Since this is considerably greater than predicted for pure water, it would appear that precipitated carbonates are involved in establishing equilibrium conditions for dissolved CO_2 and bicarbonates in 10% sludge. Gas production from substrates with acidic groups is a function of whether or not these groups are protonated, as illustrated below.

$$CH_3COOH \longrightarrow CH_4 + CO_2$$

$$CH_3COO^- + H^+ \longrightarrow CH_4 + CO_2$$

$$HCO_3^- \leftarrow H_2CO_3 \leftarrow H_2O$$

In some experiments, the pH of stock solutions of benzoic acid or phthalic acid was increased to maximize their solubility in water for dispensing into serum bottles.

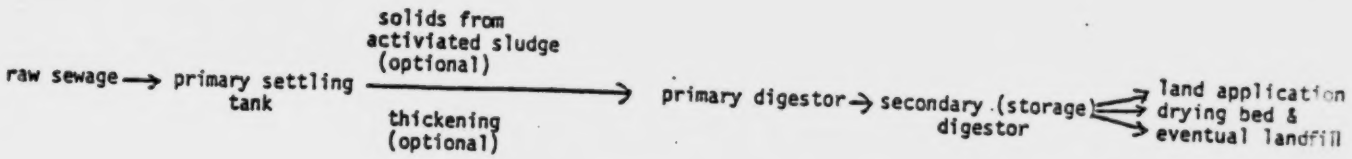
All theoretical gas production data have been corrected for CH_4 and CO_2 solubilities and where applicable, deprotonation effects. Theoretical gas production from compounds whose biodegradation we have studied is tabulated below:

Substrate		Theoretical gas production (ml)
p-Cresol	6.4 ml CH_4 + 4.1 ml CO_2 x.95 .37 6.1 1.5	7.6
Monobasic phthalate	4.9 ml CH_4 + 4.3 ml CO_2 x.95 .37 4.6 1.6	6.2
Dibasic phthalate	4.9 ml CH_4 + 3.0 ml CO_2	5.7
Di-n-butylphthalate	5.9 ml CH_4 + 4.6 ml CO_2	7.6
Ethanol	7.9 ml CH_4 + 2.6 ml CO_2	8.5
Phenol	6.1 ml CH_4 + 4.4 ml CO_2	7.4
Benzoate	5.6 ml CH_4 + 3.4 ml CO_2	6.5
m-Chlorobenzoic acid	5.25 ml CH_4 + 5.25 ml CO_2	6.9

Appendix II

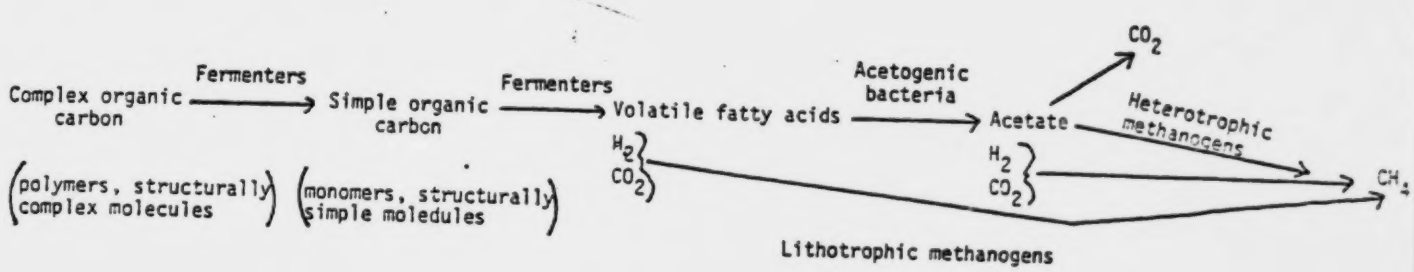
Characteristics of Sewage Treatment Plants and Anaerobic Sewage Sludge

Anaerobic digestion is only one of several methods for treating raw sewage. As such not all waste treatment plants have anaerobic sludge. Anaerobic digestion is used to stabilize raw solid waste, i.e., render it safe for handling and disposal. The flow of sewage through anaerobic digestors is illustrated in the following simplified diagram:



The primary anaerobic digestor is where principal degradation and stabilization of organic matter occurs. Primary digestor(s) are heated (between 85-100°F) and mixed to provide a consistent environment for digestion to occur. From the primary digestor, sludge is pumped to secondary digestor(s) for storage. Secondary digestors are not heated (not necessary since stabilization has already occurred) or mixed. Solids are allowed to settle; then, periodically, supernatant is pumped back through the plant while solids are disposed of either by land application or landfill.

The flow of carbon in methanogenic fermentations can be diagrammed as follows:



In considering the overall viability of sludge, one must be concerned with at least three groups of organisms: a group of organisms loosely referred to as fermenters which is responsible for breaking down more complex organic molecules into volatile fatty acids, H_2 and CO_2; the fatty acid degraders, which are responsible for degrading short chain volatile fatty acids (propionate, butyrate, etc.) into acetate, H_2, and CO_2; and the methanogens (heterotrophic and lithotrophic) which produce CH_4. The loss of viability of any one component of the consortium will result in a cessation of methanogensis.

Primary sludge, because of heating and mixing, is generally consistent over time and is suitable for use in this method. Secondary anaerobic sludge is not suitable because of greater heterogeneity and because digestor temperatures can drop to 40-50°F during winter months. Also, due to long retention times, bacterial viability is likely to be a problem.

The parameters most important in characterizing sludge are retention time, pH, total solids (drying at 100°C), and volatile solids (ashing at 550°C). The minimum retention time required for complete digestion of sewage (greatest gas production) at 35°C is approximately 15 days (16). Retention times for sludges used in our survey varied from 17 to 38 days. Total solids of sludges vary depending on whether or not solids from the primary settling tank are prethickened before pumping into the digestors, and whether or not flocculant from activated sludge is pumped into the digestor. In our sludges, total solids varied from 1.7% to 5.5% (excluding Leslie). Volatile solids is a measure of organic matter present in the sludge and is useful in estimating the degree of digestion which has occurred, however, depending on the input of nonbiodegradable organic matter (eg., lignin) volatile solids can vary. Volatile solids, as percent of total solids in our sludges, varied from 49% to 57% (excluding Leslie). The pH of the digestor indicates whether or not fermentation is coupled with methanogenesis. If the temperature drops or digestors are overloaded they can go "sour" ie., the rate of fermentation to fatty acids exceeds the rate of methanogenesis and the pH drops. This pH drop will destroy methanogenic activity. The optimum pH for digestion is approximately 6.8-7.2.

Sewage treatment operators routinely determine each of these parameters for their digestors such that they should be readily available to any potential user.

Based on our experience with sludge we recommend a retention time of 15-30 days, pH of 6.8-7.2 and a percent organic matter content of approximately 1.0-2.0 (total solids X volatile solids). This should result in good quality sludge and based on correlation between CH_4 production and percent organic matter (see below) a background CH_4 production in control bottles of 10-25 ml.

Sludge[a]	Background CH_4 production[b]	Percent organic matter (total solids X volatile solids)	
Adrian[c]	23.3	1.53	
Jackson	21.7	1.99	
Mason	19.7	1.73	
Ionia	18.0	1.82	
St. Johns	14.2	1.47	r = 0.85
Portland	12.9	1.19	
Chelsea	11.7	0.98	
Holt	8.4	0.89	

[a]Data for Pontiac, Leslie, Ann Arbor, and Charlotte sludges are not reported due to lack of data or known irregularities in digestor operation.
[b]Data from sludge survey.
[c]If Adrian is dropped r = 0.97

A more thorough treatment of theory and practice of anaerobic digestion can be found in EPA publication 625/1-79-011: Process Design Manual, Sludge Treatment and Disposal. Municipal Environmental Research Laboratory. Cincinnati, Ohio 45268.

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Figure 1. Survey of organic compounds for biodegradation in 10% sludge.

Substrate	Adrian			Jackson		
	Lag time (weeks)	Percent degradation[a]	incubation (weeks)	Lag time (weeks)	Percent degradation	incubation (weeks)
Benzoic acid	1	102[b]	4	1	96	4
o-Hydroxybenzoic acid	4	99	8	2	86	8
p-Hydroxybenzoic acid	2	115	4	2	133	4
3,4-Dihydroxy benzoic acid	3	89	8	3	80	8
p-Methoxybenzoic acid	1	106	8	1	91	8
3-Methoxy, 4-hydroxy benzoic acid	1	103	4	1	106	4
o-Aminobenzoic acid	1	104	8	1	127	8
p-Aminobenzoic acid	6	61	10	8	60	10
m-Chlorobenzoic acid	5	68	10	8	47	10
Benzyl alcohol	2	103	8	1	100	8
4-Hydroxybenzyl alcohol		0		3	61	8
Propionanilide	2	31	10	3	29	10
Phthalic acid	5	99	8	4	100	8
Di-methylphthalate	1	88	8	1	58	8
Di-ethylphthalate		0		4	32	8
Di-n-butylphthalate	2	32	4	3	85	4
Butylbenzylphthalate		0		4	24	8
Phenol	2	91	4	2	99	4
m-Methylphenol	4	92	6	5	90	6
p-Methylphenol	4	51	4	3	121	4
1,2-Dihydroxybenzene	3	98	4	3	91	4
1,3-Dihydroxybenzene	3	98	4		0	
1,2,3-Dihydroxybenzene	1	102	4	1	97	4
1,3,5-Dihydroxybenzene	1	126	4	1	170	4
Diethylene glycol	1	158	4	1	162	4
Polyethylene glycol-1000	1	155	4	1	180	4
Polyethylene glycol-20,000	2	112	4	2	50	4
Geraniol	4	73	8	4	72	8
Amyl alcohol	1	95	7	1	100	7
1-Octanol	1	110	7	1	88	7
2-Octanol		0		7	65	14
2-Hexanone		0		7	87	12
Acrylic acid	2	92	8	2	83	8
Ethylacetate	1	91	8	1	88	8
3-Hydroxy-2-butamone	1	90	7	1	82	7
2,3-Butanediol	1	111	7	1	75	7
Acetylsalicylic acid	1	93	8	2	108	8
4-Hydroxyacetanilide	2	27	9	2	39	9
4-Chloroacetanilide	2	18	4	2	25	4

[a]Percent theoretical methane production.

[b]Values are approximate. Average of two determinations.

Figure 2. Compounds for which we have no evidence of degradation in 10% sludge.

Chloronaphthol (4)[a]	2-Hydroxybenzyl alcohol (8)
DDT (4)	Tricresylphosphate (8)
PBB (4)	Cresyldiphenylphosphate (8)
Phenoxyacetic acid (4)	o-Aminophenol (8)
Methyl-IDA (4)	p-Aminophenol (8)
IDA (4)	3-Methylsalicylic acid (8)
N-methylacetanilide (4)	Orcinol (8)
5-Chloro-2-hydroxyaniline (4)	4-Methylcatechol (8)
2,6-Diethylaniline (4)	2-Methylresorcinol (8)
3,5-Dichloroaniline (4)	4-Chlorocatechol (8)
p-Chloroaniline (4)	m-Aminobenzoic acid (8)
NTA (8)	o-Chlorobenzoic acid (8)
Di-ethylhexylphthalate (8)	p-Chlorobenzoic acid (8)
Di-octlylphthalate (8)	3,4-Dichlorobenzoic acid (8)
4-Aminoacetophenone (8)	Benzene (8)
4-Chlororesorcinol (8)	Toluene (8)
o-Chlorophenol (8)	o-Methylbenzoic acid (8)
Acrolein (8)	m-Methylbenzoic acid (8)
Acrylamide (8)	p-Methylbenzoic acid (8)
Dimethylformamide (8)	2-Amino-5-chlorobenzoic acid (8)
Naphthol (8)	3-Chloro-4-hydroxybenzoic acid (8)
2-Hexene (8)	Benzylacetone (8)
3-Hexanol (8)	o-Cresol (8)
2-Hexanol (8)	Anisole (8)
3-Henanone (8)	

[a]Number in parenthesis indicates weeks of incubation.

Figure 3. Compounds which were inhibitory to methanogenesis in 10% sludge.

Substrate	Percent inhibition[a]	
	Adrian	Jackson
m-Chlorophenol	14	58
p-Chlorophenol	7	41
5-Chlorosalicylic acid	78	85
p-Nitrophenol	97	97
Chloroform	98	96
Pentachlorophenol	94	84
Hexachlorocyclopentadiene	88	95
Trichloroacetic acid	96	98
4-Phenoxybutyric acid	64	90
Atrazine	76	80

[a]After a 4 week incubation

Figure 4. Extraction efficiencies for phthalate esters, tri-m-cresylphosphate, and monochlorophenols in whole sludge.

Compound	Percent recovery		
	Jackson	Holt	Adrian
Di-n-methylphthalate	40	40	49
Di-n-ethylphthalate	57	41	64
Di-n-butylphthalate	97	69	102
Butylbenzylphthalate	101	91	94
Di-(2-ethylhexyl)phthalate	116	109	100
Di-n-octylphthalate	103	127	106
Tri-m-cresylphosphate	98	88	102
o-Cresol	69	61	
m-Cresol	72	67	
p-Cresol	65	51	
o-Chlorophenol	74	69	
m-Chlorophenol	84	80	
p-Chlorophenol	73	76	

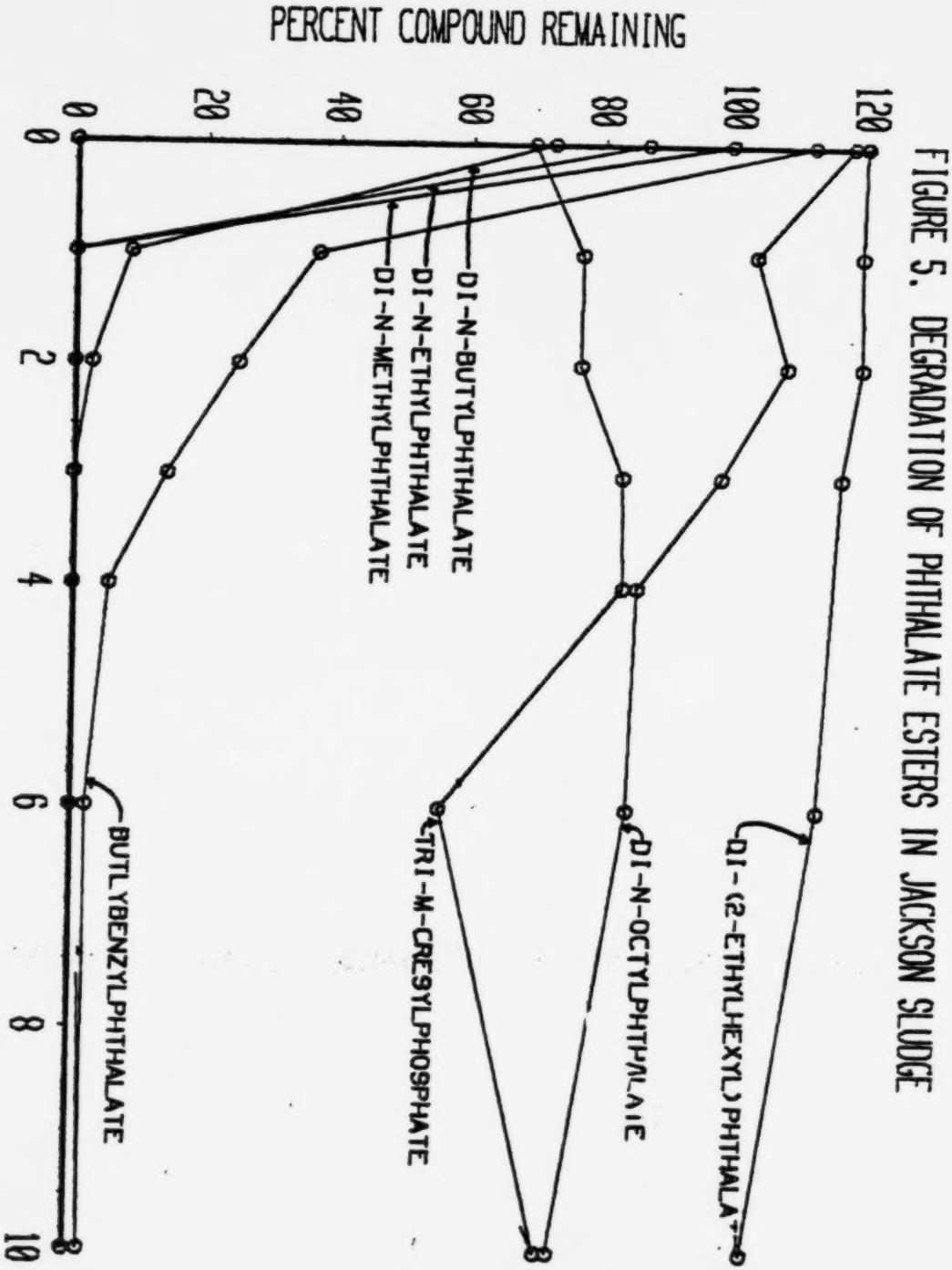


FIGURE 5. DEGRADATION OF PHTHALATE ESTERS IN JACKSON SLUDGE

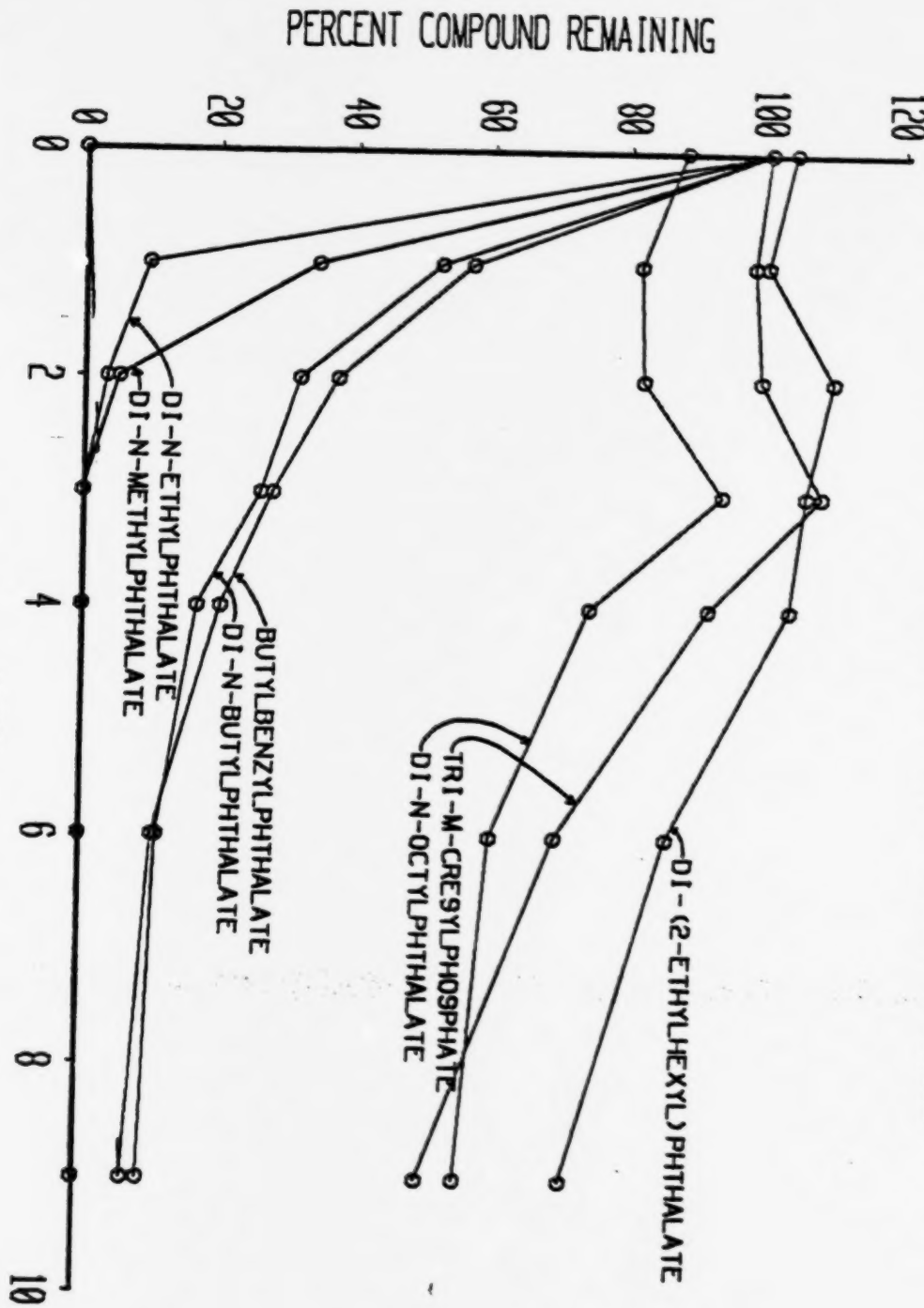


FIGURE 6. DEGRADATION OF PHTHALATE ESTERS IN ADRIAN SLUDGE

FIGURE 7 DEGRADATION OF PHTHALATE ESTERS IN HOLT SLUDGE

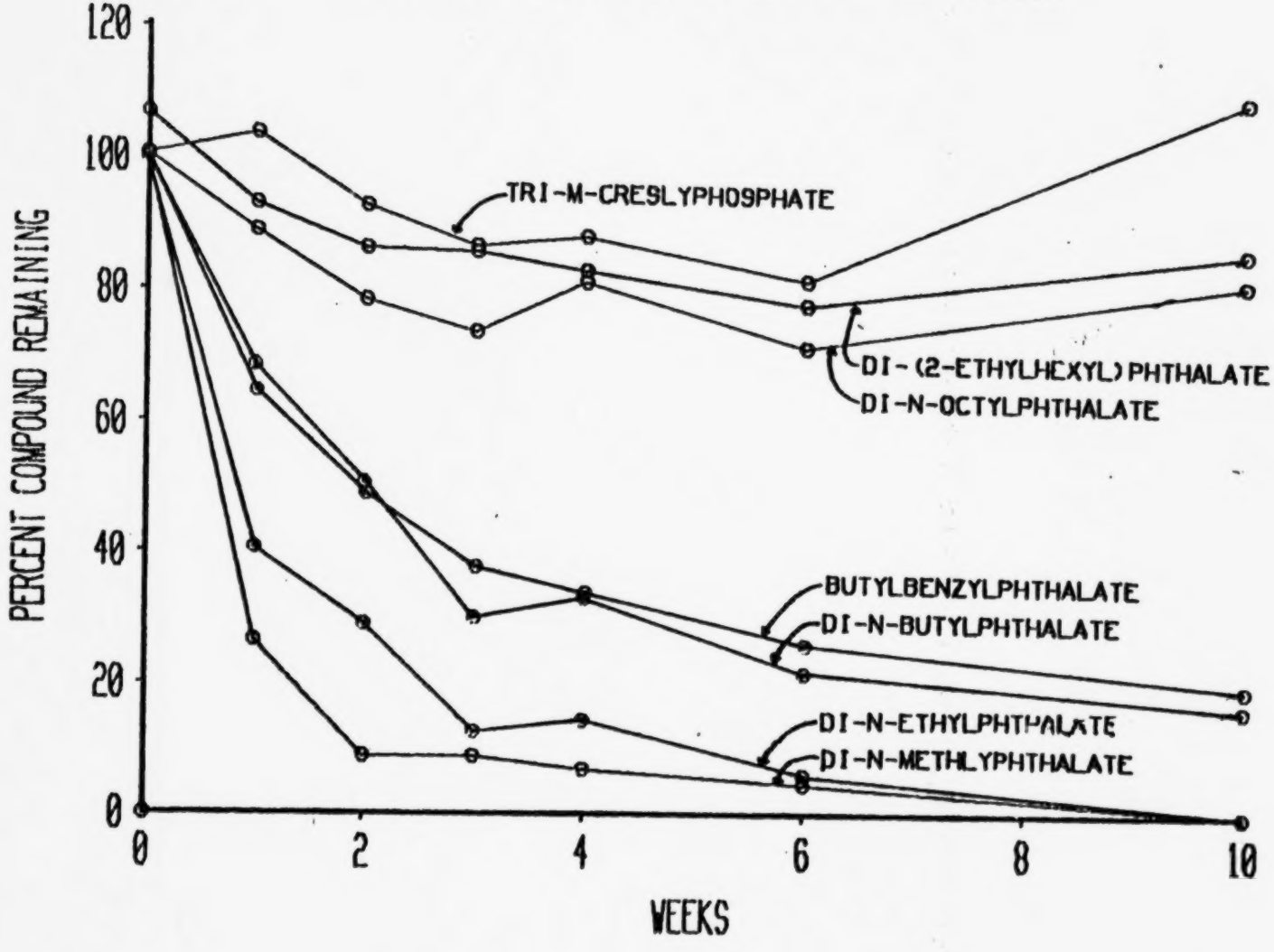


Figure 8. Comparison of degradation measured by methane production vs substrate disappearance in Adrian and Jackson sludge.

	Adrian		Jackson			
Phthlate esters	CH_4 production in 10% secondary sludge	Substrate disappearance in whole sludge	CH_4 production in 10% secondary sludge	CH_4 production in 10% primary sludge	Substrate disappearance in whole sludge	Substrate disappearanc in 10% sludg
Di-n-methyl	88[a]	100[b]	58[a]	82[a]	100[b]	100[b]
Di-n-ethyl	0	100	32	76	100	100
Di-n-butyl	32	90	85	80	100	100
Butylbenzyl	0	93	24	103	98	100
Di-(2-ethylhexyl)	0	28	0	9	0	-
Di-n-octyl	0	44	0	13	27	-

[a]Percent of theoretical methane production

[b]Percent disappearance

FIGURE 9. PHTHALATE ESTER DEGRADATION IN 10% JACKSON SLUDGE

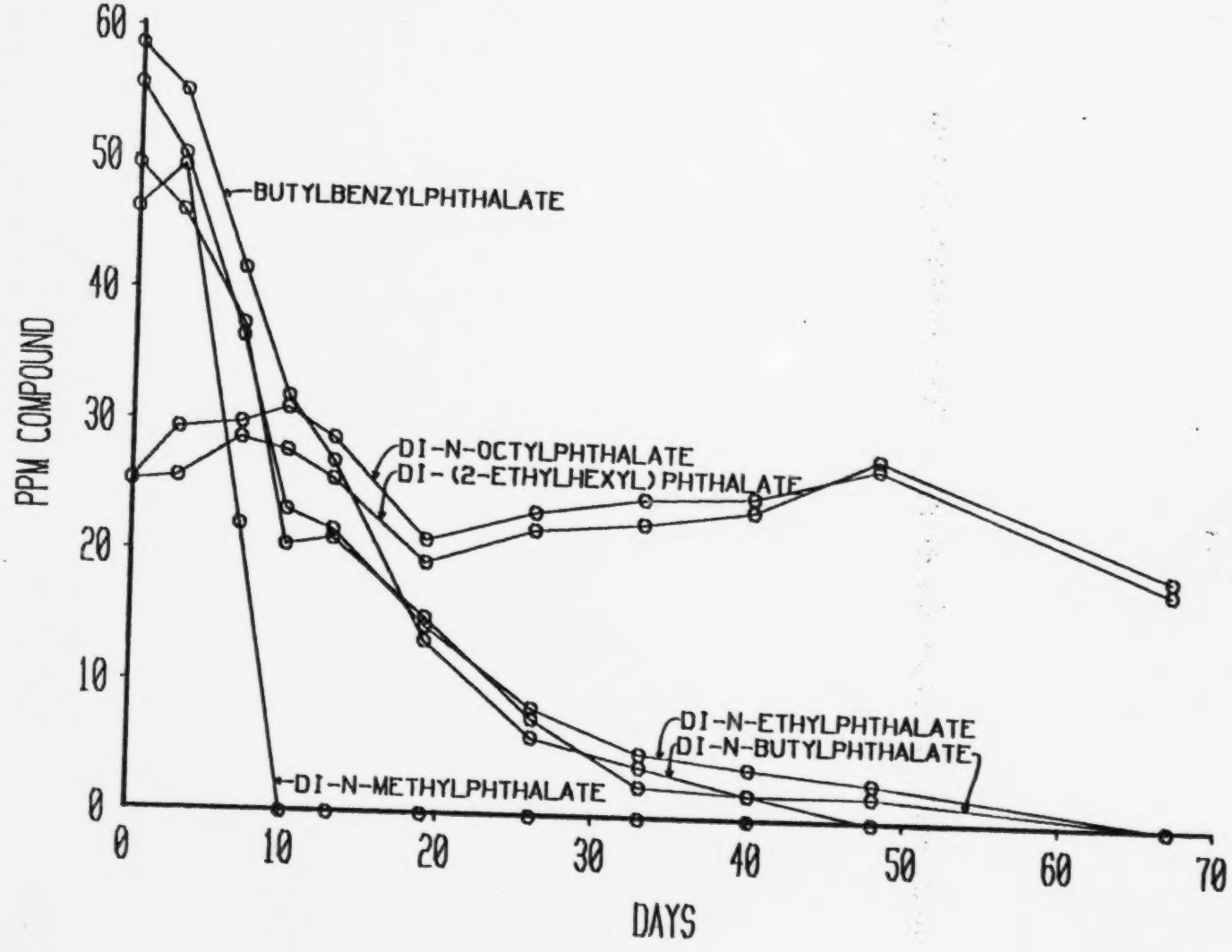


FIGURE 10. PHTHALATE ESTER DEGRADATION IN 10% JACKSON SLUDGE

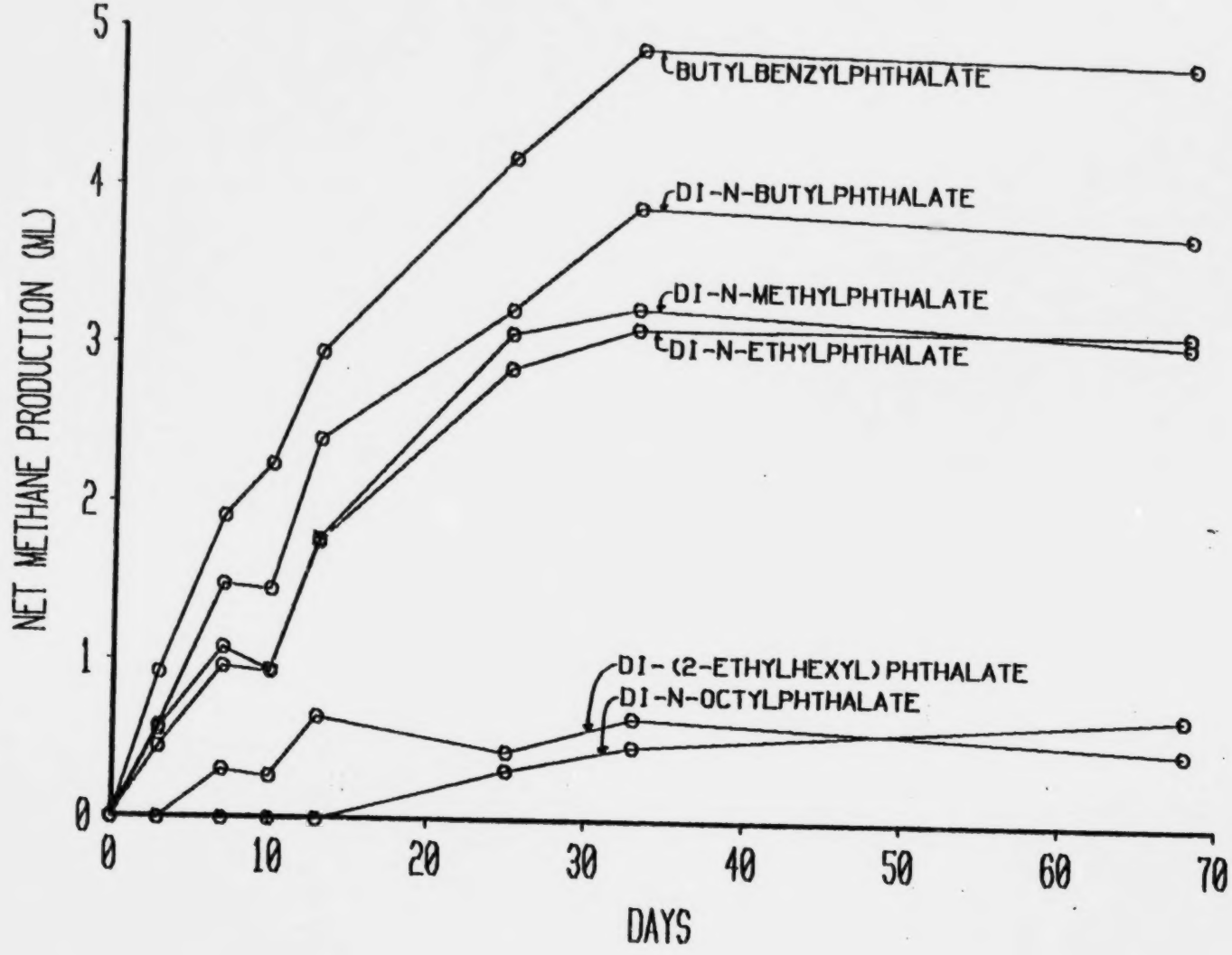


FIGURE 11. DEGRADATION OF CRESOLS AND MONOCHLOROPHENOLS IN JACKSON SLUDGE

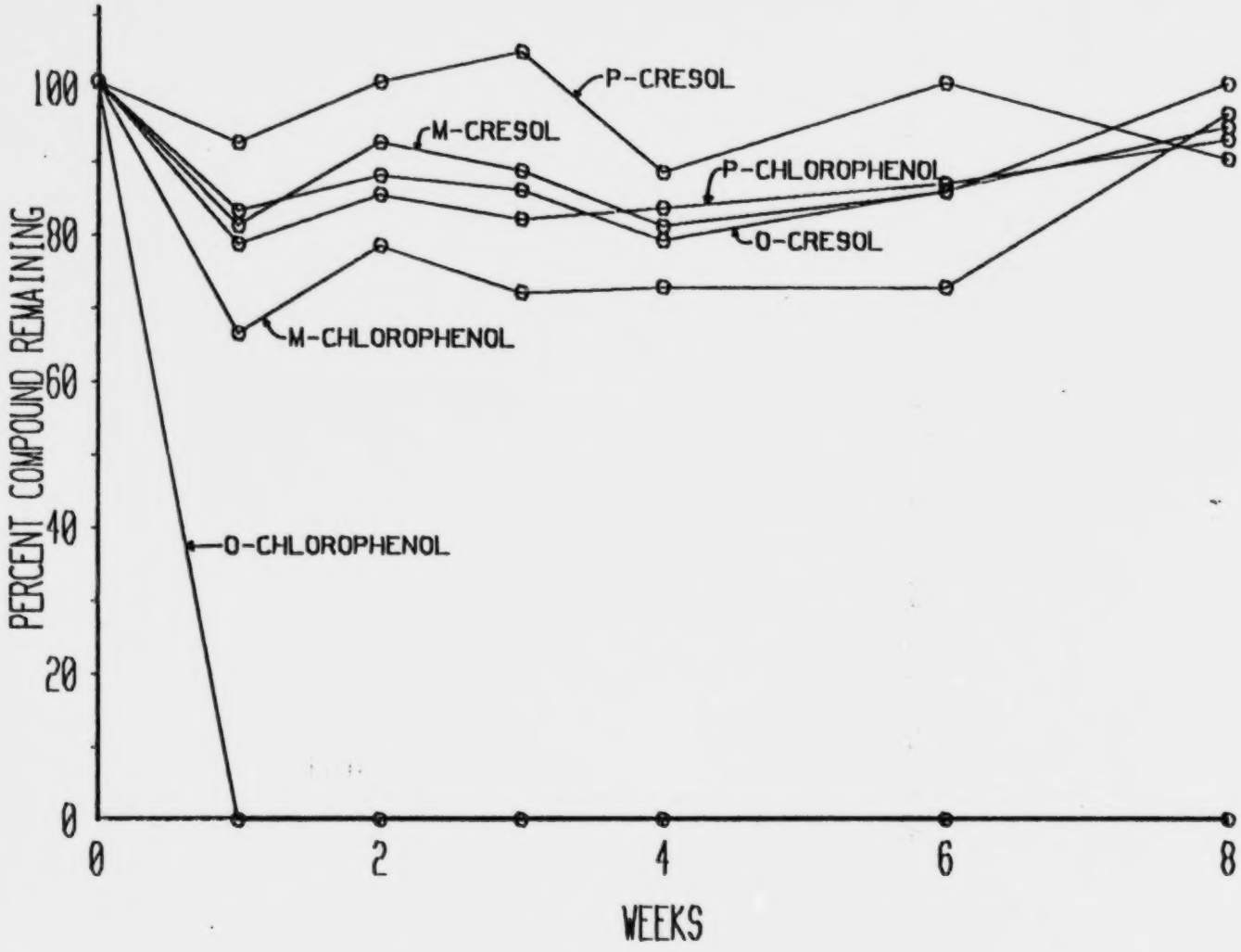


FIGURE 12 . DEGRADATION OF CRESOLS AND MONOCHLOROPHENOLS IN HOLT SLUDGE

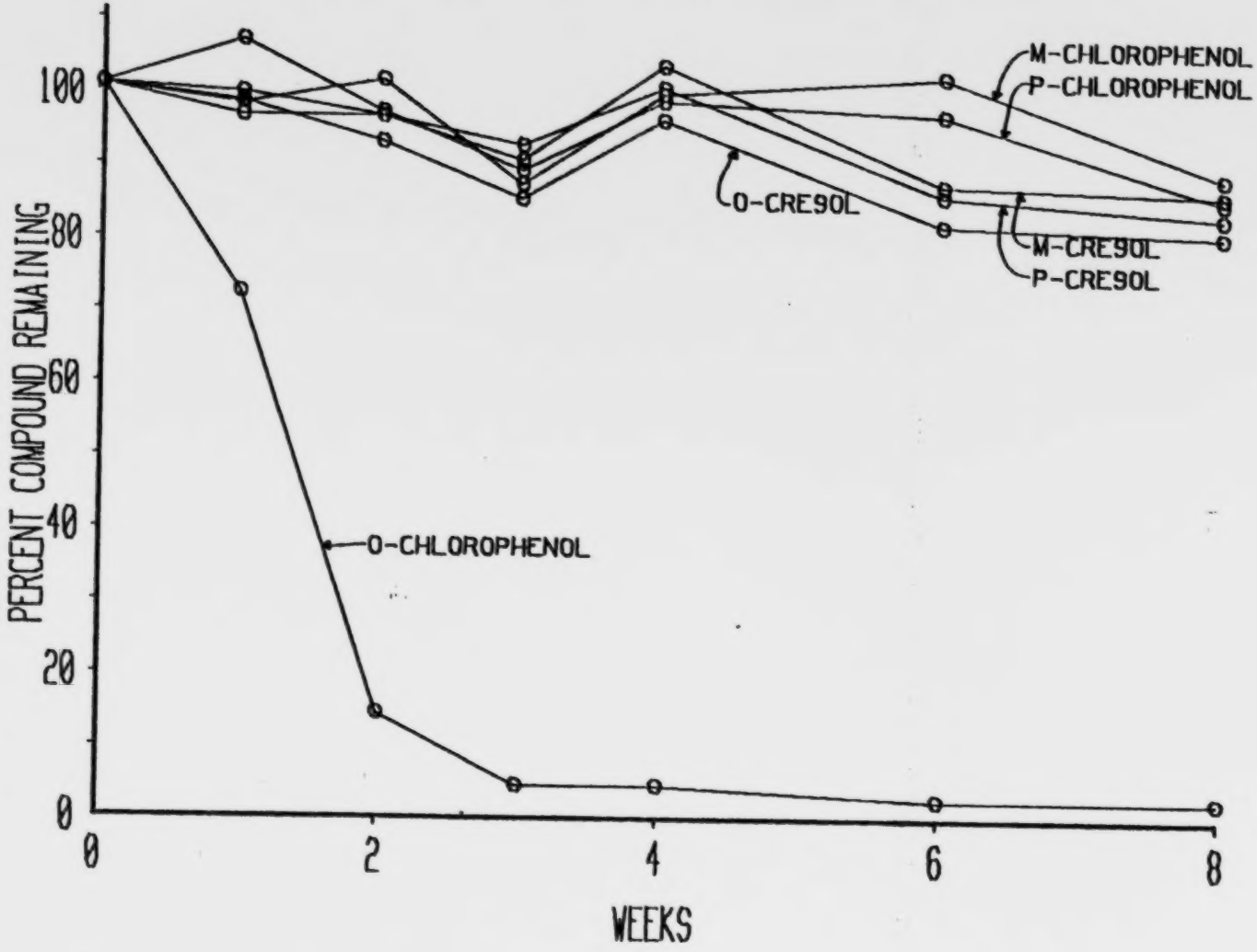


Figure 13. Characterization of sludges.

City	Percent industrial input	Number of people served	Flow (10^6gal/day)	Total solids(%)	Volatile solids(%)	Average retention time (days)
Adrian	17.5	20,410	5.0	2.8	55	20
Jackson	17.1	60,000	17.0	4.0	55	20
Ann Arbor	2.0	95,260	15.0	4.0	55	
Pontiac	39.7	92,170	20.0	n.d.	n.d.	
St. Johns	28.3	5,200	1.0	3.0	49	20
Ionia	34.9	5,200	1.2	3.5	52	17
Holt	0.0	17,000	1.1	1.7	57	38-39
Mason	19.4	1,170	0.8	3.8	50	21
Leslie	0.0	2,040	0.2	10.2	40	
Chelsea	18.0	3,950	0.4	1.8	56	30
Charlotte	4.2	8,200	1.2	4.3	55	
Portland	13.6	3,500	0.6	2.3	50	19

[a]Calculated by dividing total known industrial input by total flow

Figure 14. Comparison of degradation results for twelve sludges.

Substrate	Adrian Lag[a] time	Adrian % deg.	Jackson Lag time	Jackson % deg.	Ann Arbor Lag time	Ann Arbor % deg.	Pontiac Lag time	Pontiac % deg.	St. Johns Lag time	St. Johns % deg.	Ionia Lag time	Ionia % deg.	Holt Lag time	Holt % deg.	Mason Lag time	Mason % deg.	Leslie Lag time	Leslie % deg.	Chelsea Lag time	Chelsea % deg.	Charlotte Lag time	Charlotte % deg.	Portland Lag time	Portland % deg.
Ethanol	(1)	62	(1)	78	(1)	86	(1)	62	(1)	78	(1)	71	(1)	33	(1)	91	(1)	39	(1)	54	(1)	55	(1)	52
Polyethylene glycol-20,000	(2)	61	(2)	43	i.d.[b]		(2)	55	(3)	78	(2)	51	(3)	53	(2)	82	(5)	42	(3)	38	0		(3)	98
p-Cresol	(4)	91	(4)	88	(4)	101	(3)	72	(4)	79	(3)	80	(2)	88	(3)	94	(5)	79	(2)	62	i.d.		(3)	77
Phthalic acid	(4)	88	(5)	80	(4)	132	(3)	74	(4)	113	(3)	73	(2)	86	(4)	96	(5)	58	(3)	60	(3)	61	(4)	96
m-Cresol	(6)	82	(4)	103	(4)	85	(4)	55	0		0		(5)	77	(4)	91	i.d.		i.d.		0		0	
Di-n-butyl phthalate	(8)	24	(5)	49	(4)	91	0		(4)	37	(2)	36	i.d.		0		0		0		0		0	
2-Octanol	0		(8)	22	(8)	58	0		0		0		0		(4)	87	0		0		0		0	
m-Chlorobenzoic acid	(4)	85	0		0		0		0		0		(6)	85	0		0		0		(4)	51	0	
Propionanilide	0		0		(4)	36	0		(5)	33	0		(8)	23	0		0		0		0		0	
o-Cresol	0		0		0		0		0		0		0		0		0		0		0		0	
Di-n-octyl phthalate	0		0		0		0		0		0		0		0		0		0		0		0	
Chloroform	<0[c]		<0		<0		<0		<0		<0		<0		<0		<0		<0		<0		<0	

[a]Lag time for approximately 20% of theoretical CH_4 production expressed in weeks.
[b]i.d. = insufficient data. Generally due to leaky bottles or only one bottle showing evidence for degradation.
[c]Inhibition of methane production greater than 95% for all sludges.

Figure 15. Effect of length of sludge storage on gas production in 10% sludge.

Length of storage	Mean percent of theoretical gas production + S.D.[a] p-Cresol	Phthalic acid	Di-n-butylphthalate	Ethanol	Background gas production (ml)
			Jackson		
Week 0	103 ± 7.8[a]	111 ± 27.4	76 ± 5.6	99 ± 13.5	44.1
Week 1	76 ± 3.8	122 ± 12.6	97 ± 22.0	88 ± 3.4	44.5
Week 2	133 ± 3.2	64 ± 25.3	109 ± 14.8	104 ± 2.4	40.2
Week 3	93 ± 12.1	96 ± 14.7	78 ± 2.5	78 ± 4.8	44.1
Week 4	105 ± 11.0	77 ± 7.7	93 ± 12.4	83 ± 6.8	43.8
Avg.	106 7.6[b]	94 17.5	91 11.5	90 6.2	
			Holt		
Week 0	112 ± 2.2	98 ± 2.6	30 ± 9.4	96 ± 16.6	17.2
Week 1	109 ± 15.1	101 ± 13.7	36 ± 10.0	88 ± 11.4	17.2
Week 2	120 ± 6.0	116 ± 16.8	57 ± 8.2	113 ± 12.3	15.2
Week 3	--	98 ± 13.6	48 ± 7.0	98 ± 17.0	14.0
Week 4	118 ± 6.3	113 ± 46.4	52 ± 10.4	107 ± 2.0	14.0
Avg.	115 7.4	105 18.6	44 9.0	100 11.9	
			Chelsea		
Week 0	83 ± 9.9	69 ± 16.1	51 ± 20.1	88 ± 18.2	6.5
Week 1	112 ± 5.2	93 ± 1.2	39 ± 4.1	123 ± 13.2	12.5
Week 2	89 ± 16.5	99 ± 10.4	22 ± 1.0	91 ± 7.3	13.6
Week 3	100 ± 18.1	111 ± 12.3	46 ± 7.7	127 ± 8.9	12.4
Week 4	132 ± 2.7	123 ± 4.8	32[c]	111[c]	8.4
Avg.	103 10.5	99 9.0	38 8.2	108 11.9	
			Adrian		
Week 0	105 ± 14.7	97 ± 24.1	37 ± 7.1	off scale[b]	60.7
Week 1	128 ± 10.5	63 ± 14.5	86 ± 37.7		62.3
Week 2	108 ± 7.1	83 ± 57.8	92 ± 8.2		59.5
Week 3	--	102 ± 25.6	98 ± 11.6		66.6
Week 4	138 ± 9.6	87[c]	41[c]		58.2
Avg.	120 10.5	86 30.5	71 16.1		

[a]Gas production within 8 weeks of incubation after the indicated length of storage prior to incubation.

[b]Background gas production plus gas from ethanol exceeded upper limit of pressure transducer.

[c]Gas production in two out of three bottles at or below controls (probably due to leaks).

FIGURE 16. EFFECT OF LENGTH OF SLUDGE STORAGE ON DEGRADATION OF P-CRESOL IN 10% HOLT SLUDGE

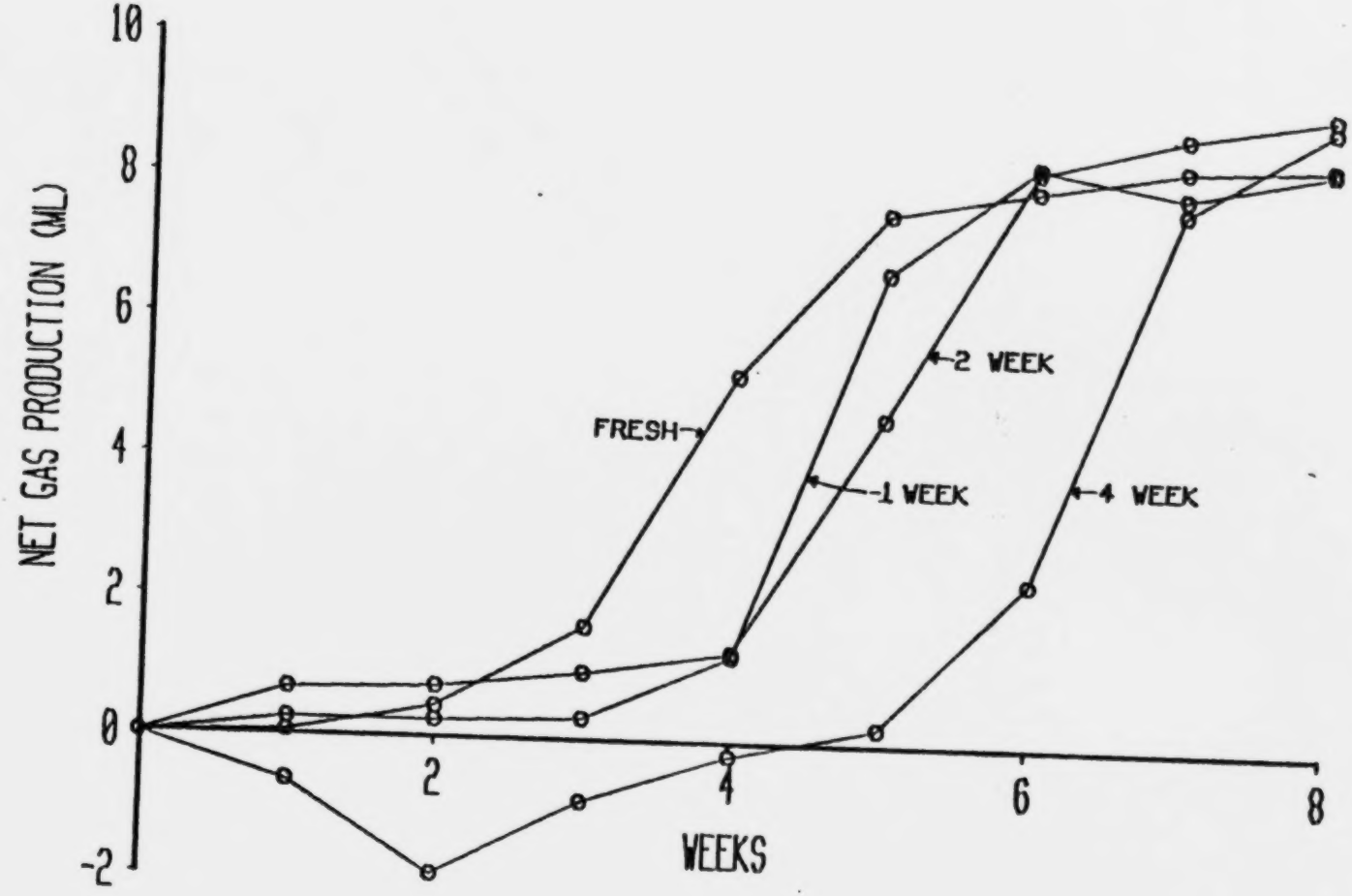


FIGURE 17. EFFECT OF LENGTH OF SLUDGE STORAGE ON DEGRADATION OF PHTHALIC ACID IN 10% HOLT SLUDGE

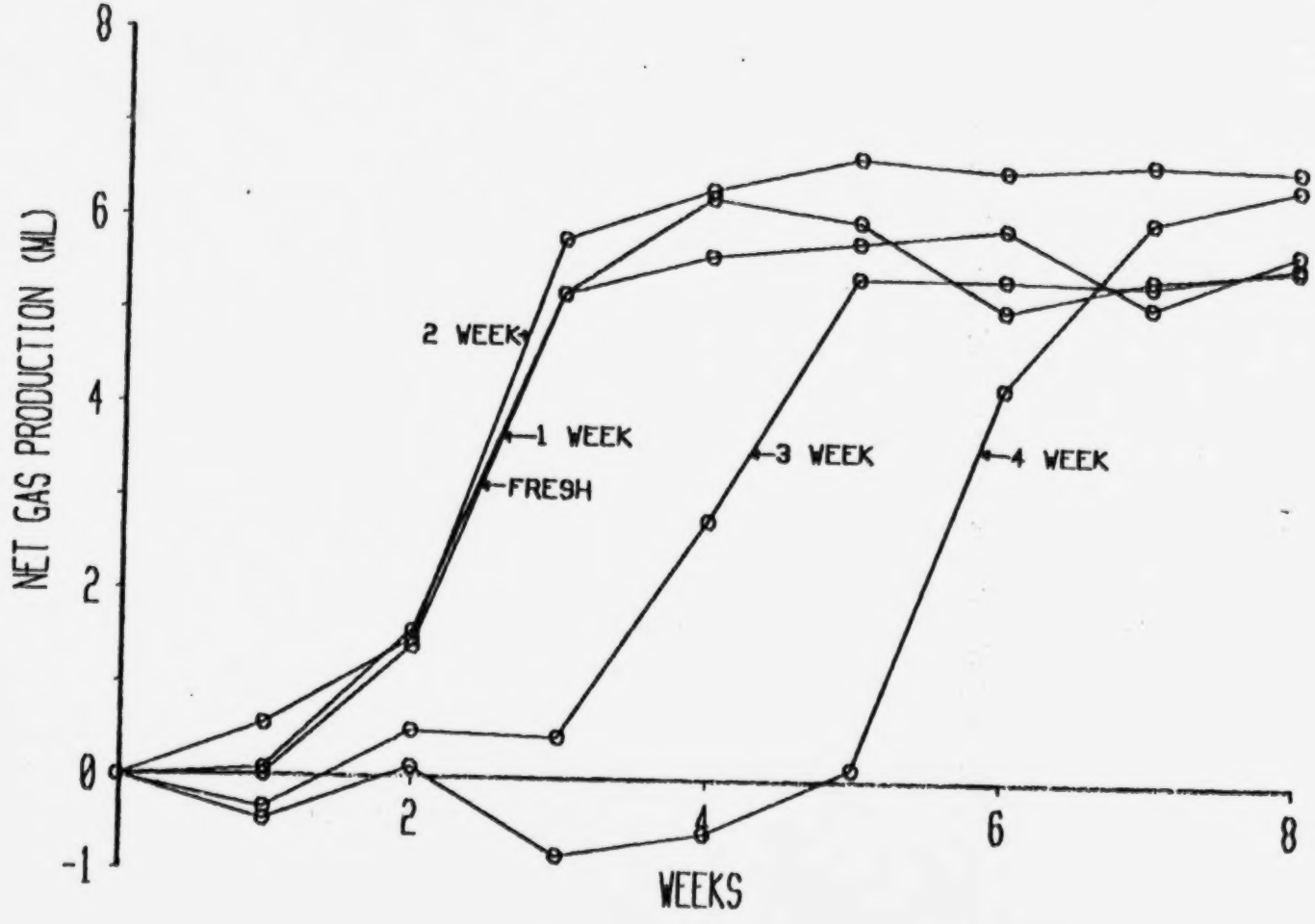


FIGURE 18. EFFECT OF LENGTH OF SLUDGE STORAGE ON DEGRADATION OF DI-N-BUTYLPHTHALATE IN 10% HOLT SLUDGE

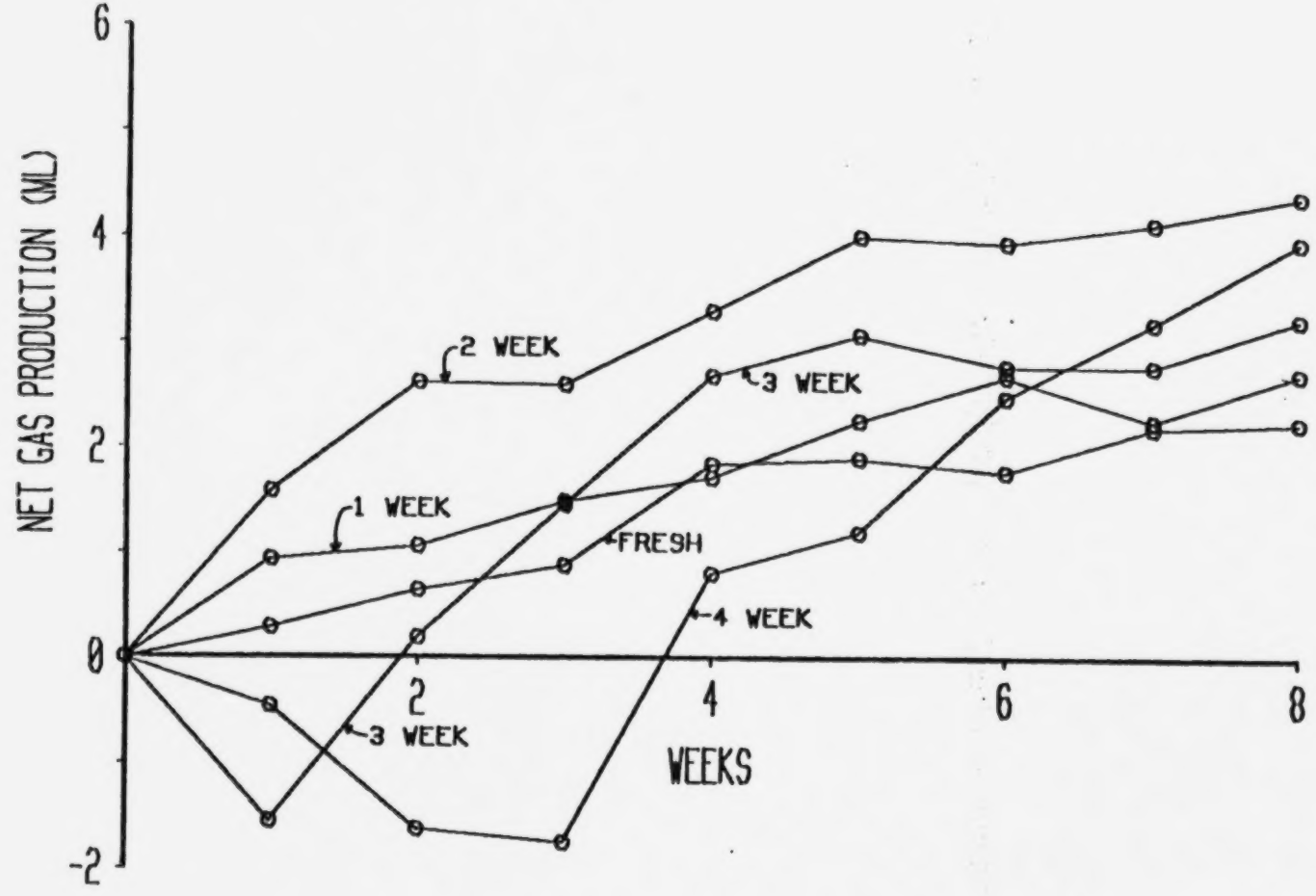


FIGURE 19. EFFECT OF LENGTH OF SLUDGE STORAGE ON THE DEGRADATION OF ETHANOL IN 10% HOLT SLUDGE

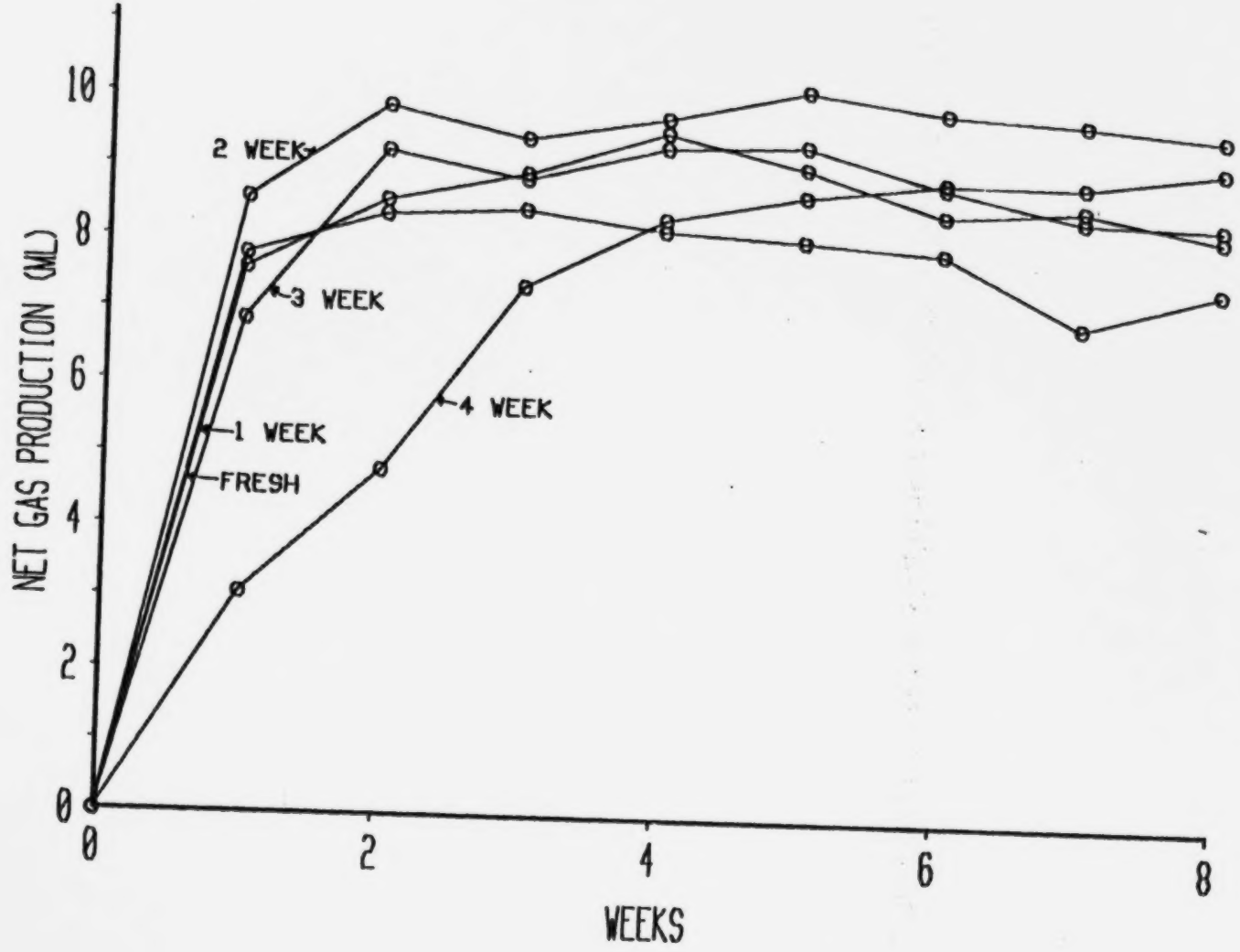


FIGURE 29. EFFECT OF LENGTH OF SLUDGE STORAGE ON DEGRADATION OF P-CRESOL IN 10% JACKSON SLUDGE

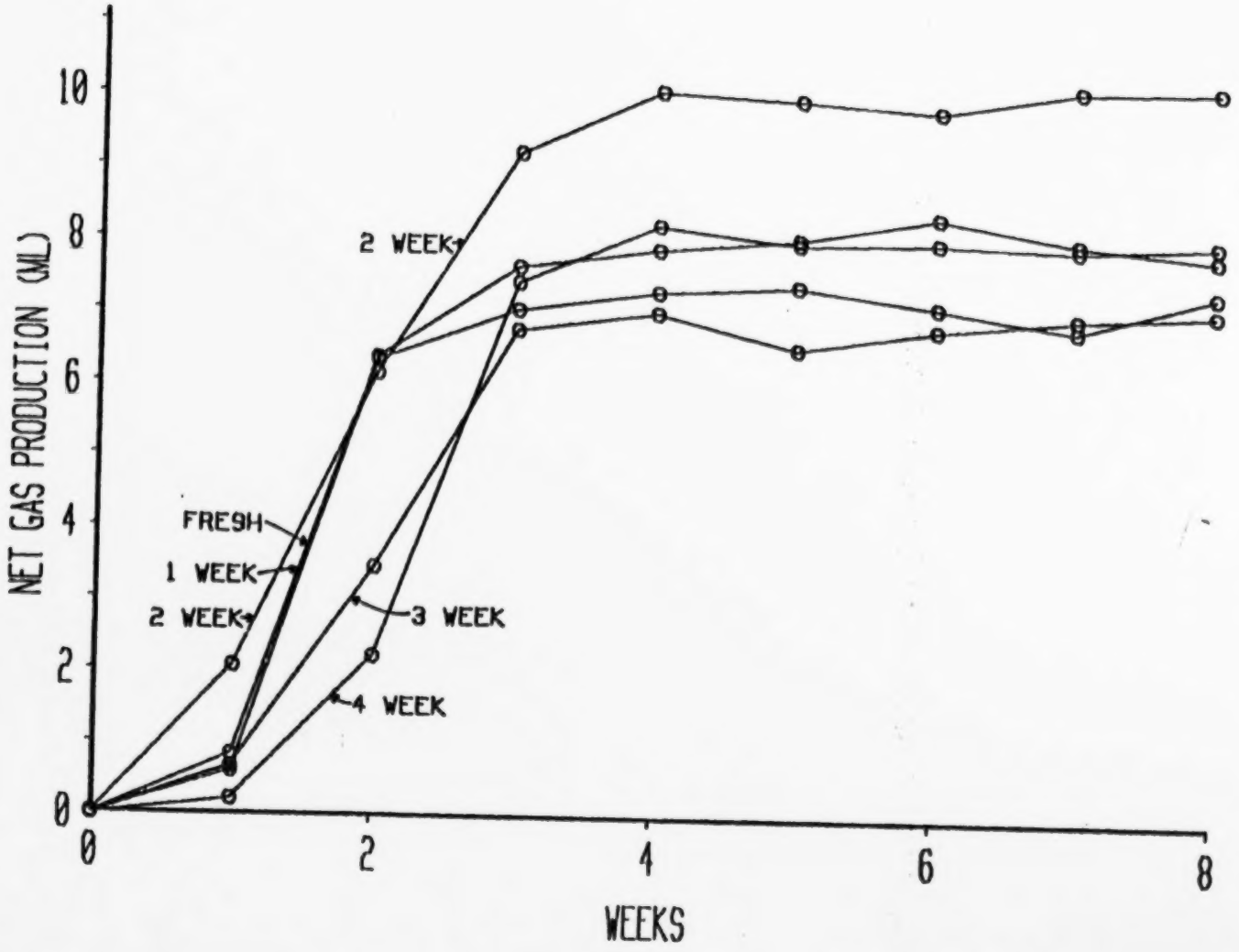


FIGURE 21. EFFECT OF LENGTH OF SLUDGE STORAGE ON DEGRADATION OF PHTHALIC ACID IN 10% JACKSON SLUDGE

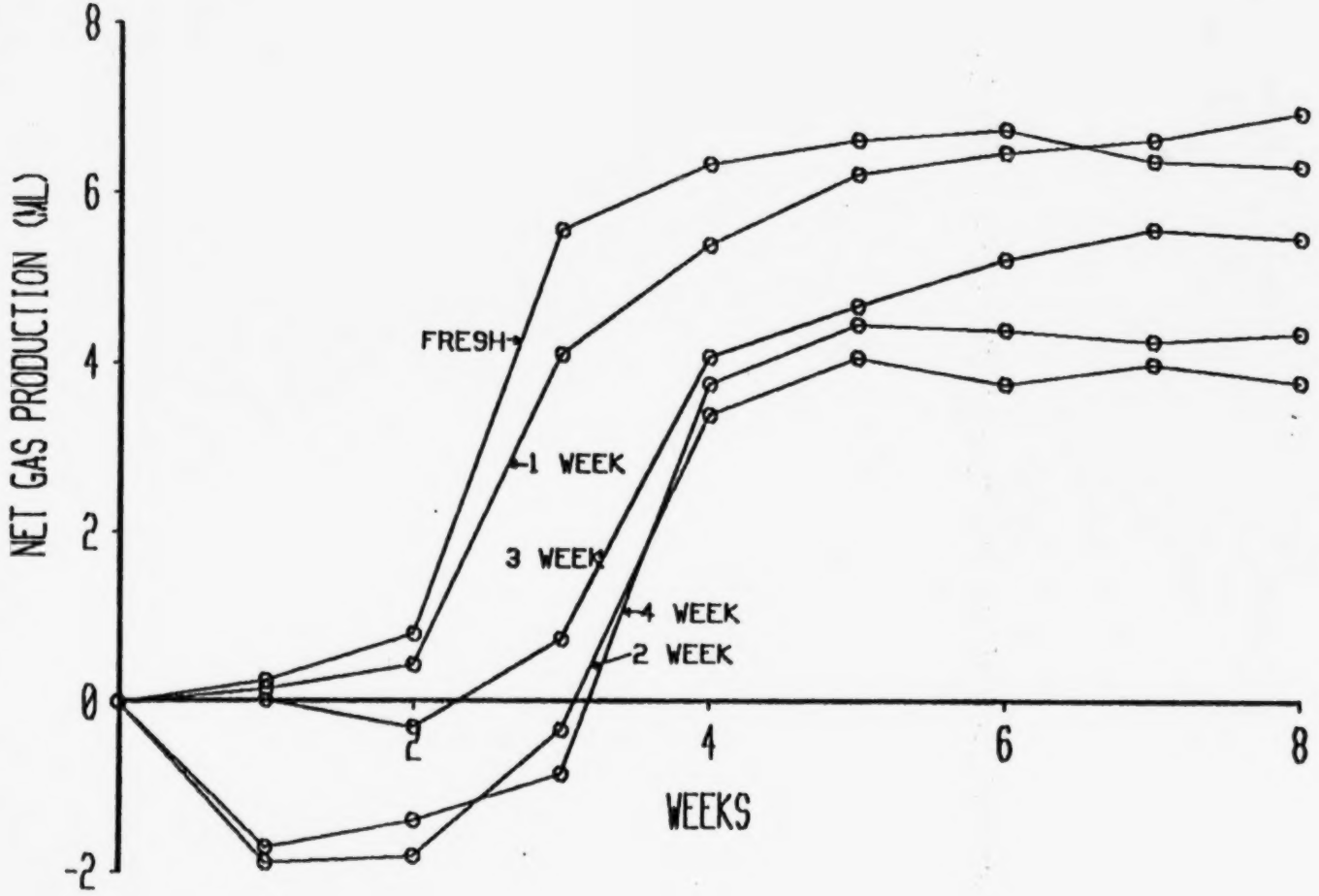


FIGURE 22. EFFECT OF LENGTH OF SLUDGE STORAGE ON DEGRADATION OF DI-N-BUTYLPHTHALATE IN 10% JACKSON SLUDGE

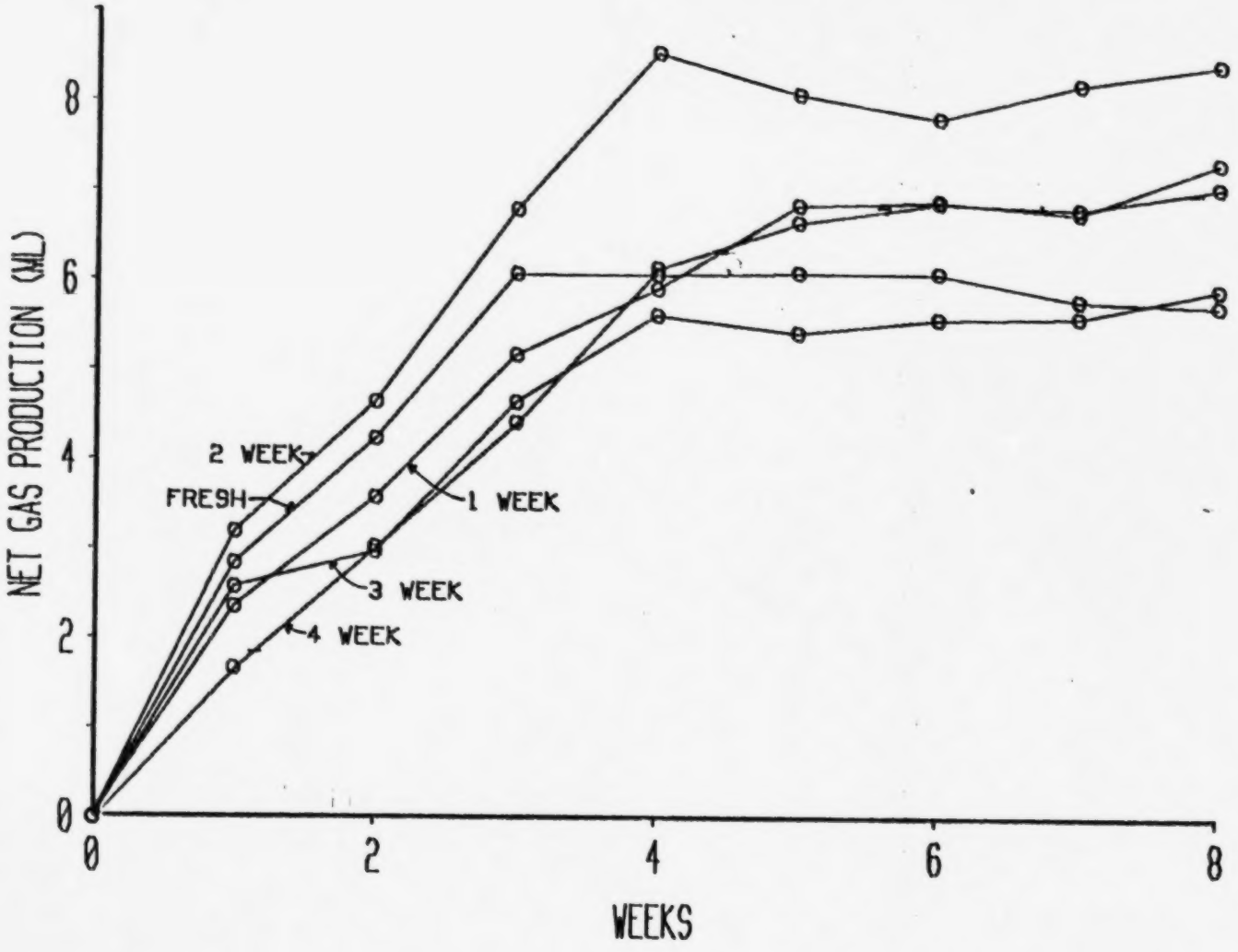


FIGURE 23. EFFECT OF LENGTH OF SLUDGE STORAGE ON THE DEGRADATION OF ETHANOL IN 10% JACKSON SLUDGE

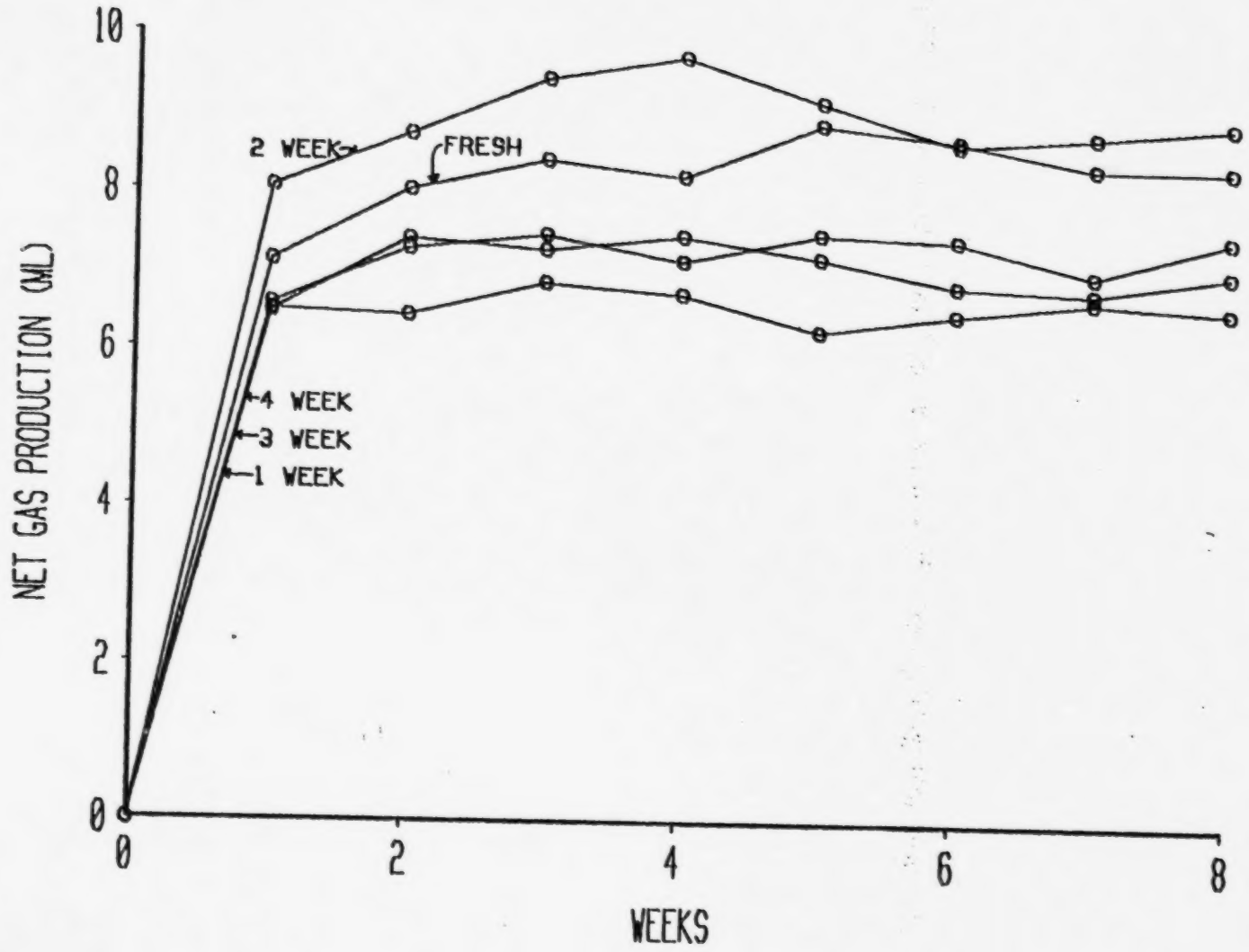


FIGURE 24. EFFECT OF SLUDGE STORAGE ON LAG TIMES FOR DEGRADATION OF P-CRESOL IN 10% SLUDGE

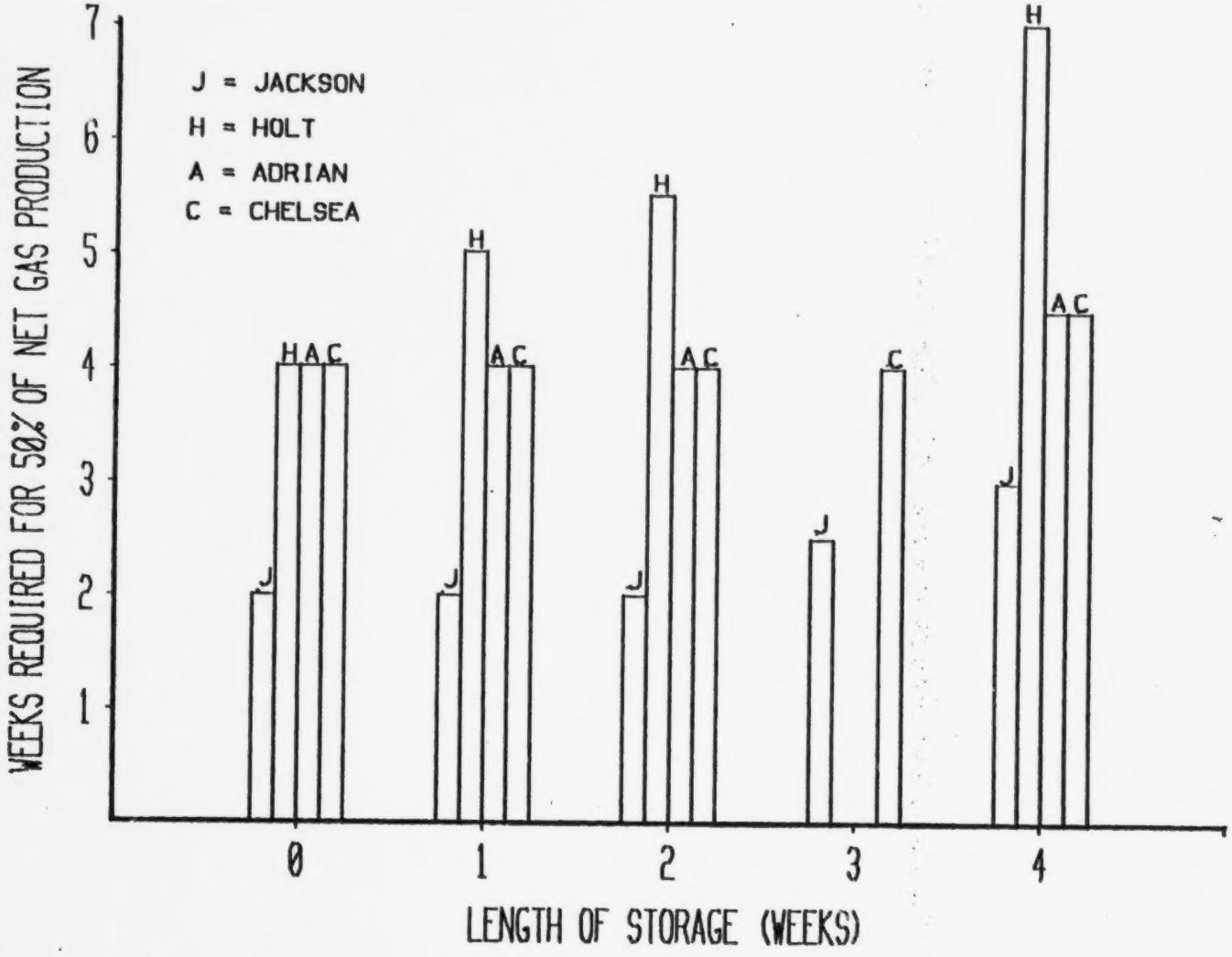


FIGURE 25. EFFECT OF SLUDGE STORAGE ON LAG TIMES FOR DEGRADATION OF PHTHALIC ACID IN 10% SLUDGE

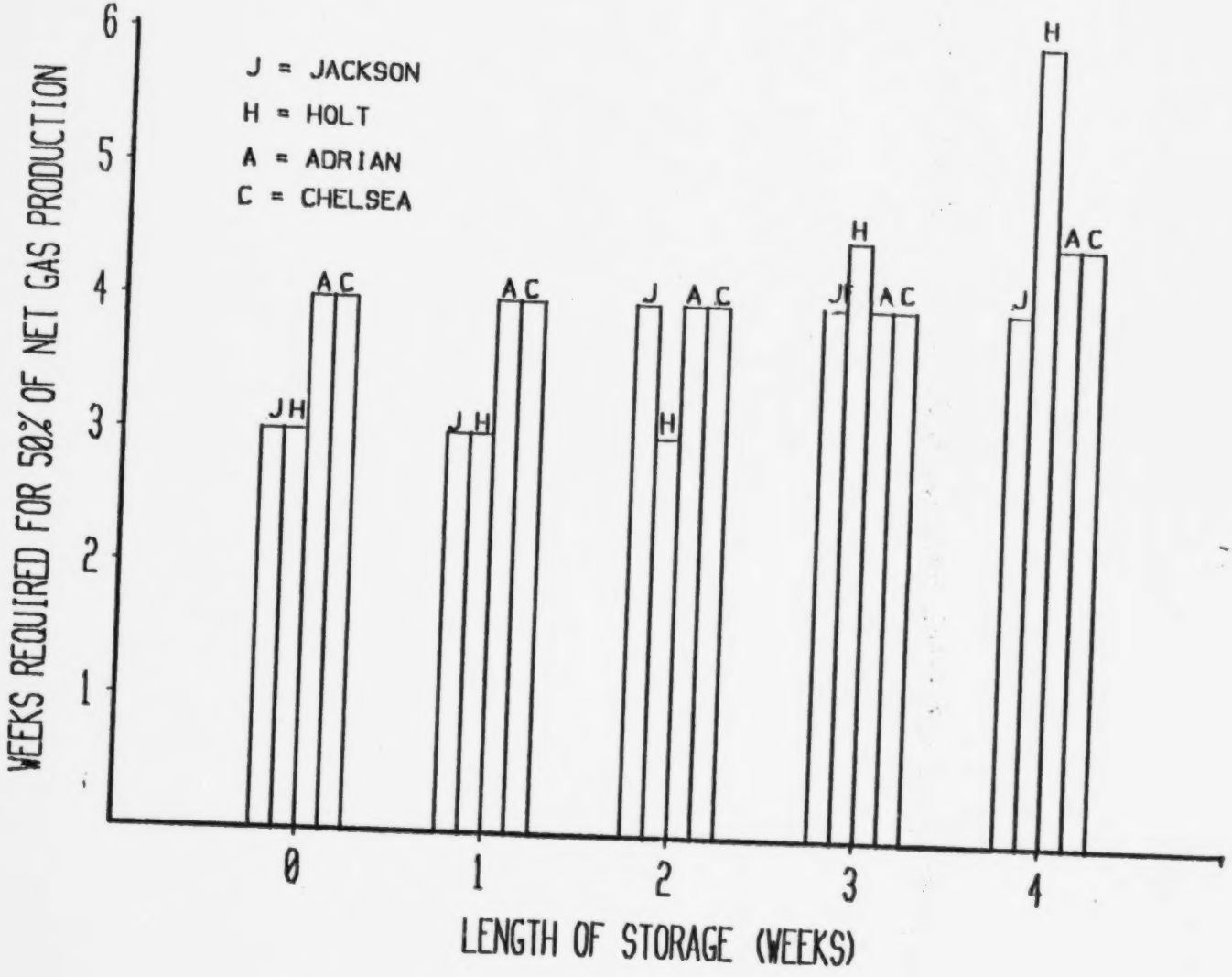
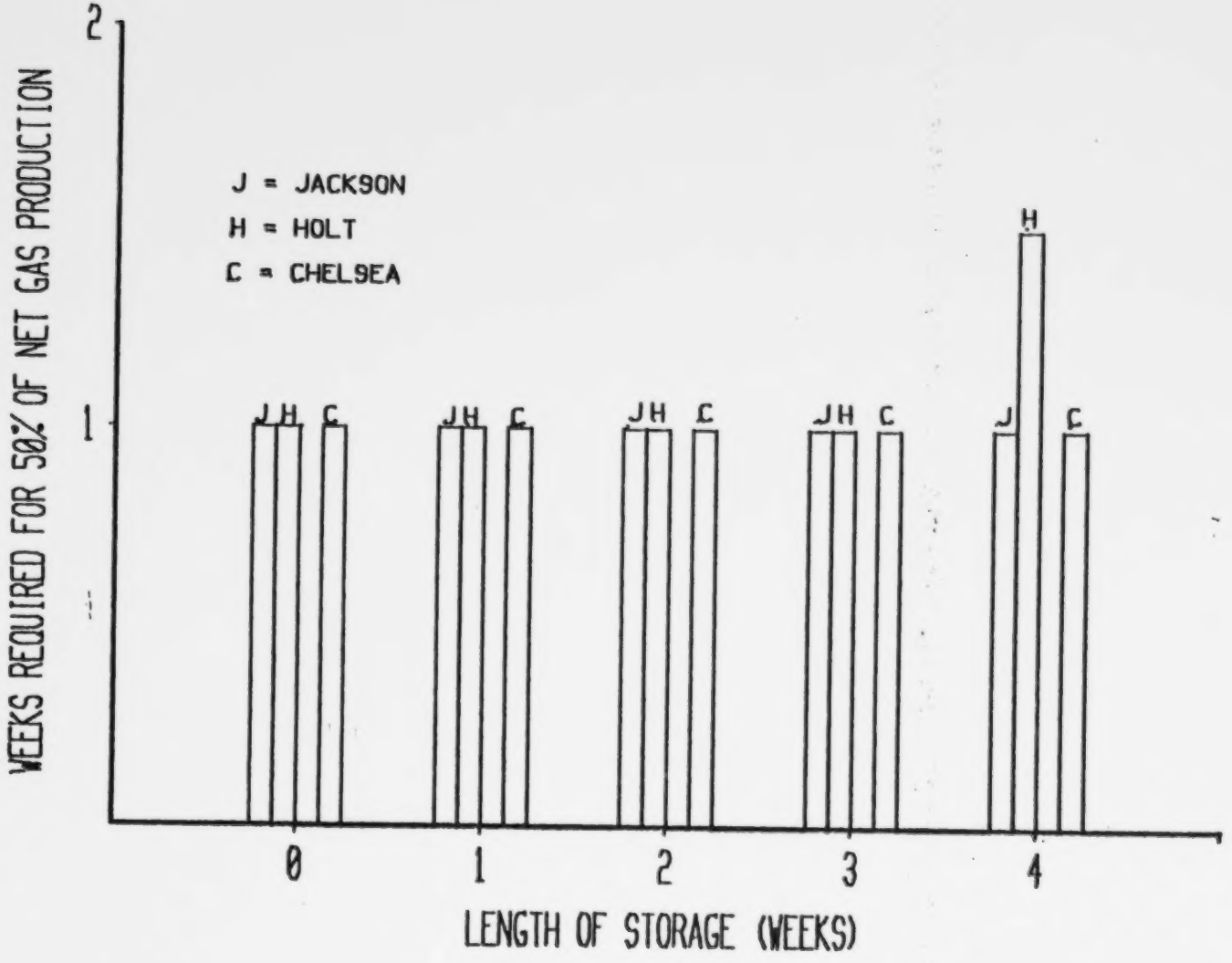


FIGURE 26. EFFECT OF SLUDGE STORAGE ON LAG TIMES FOR DEGRADATION OF ETHANOL IN 10% SLUDGE



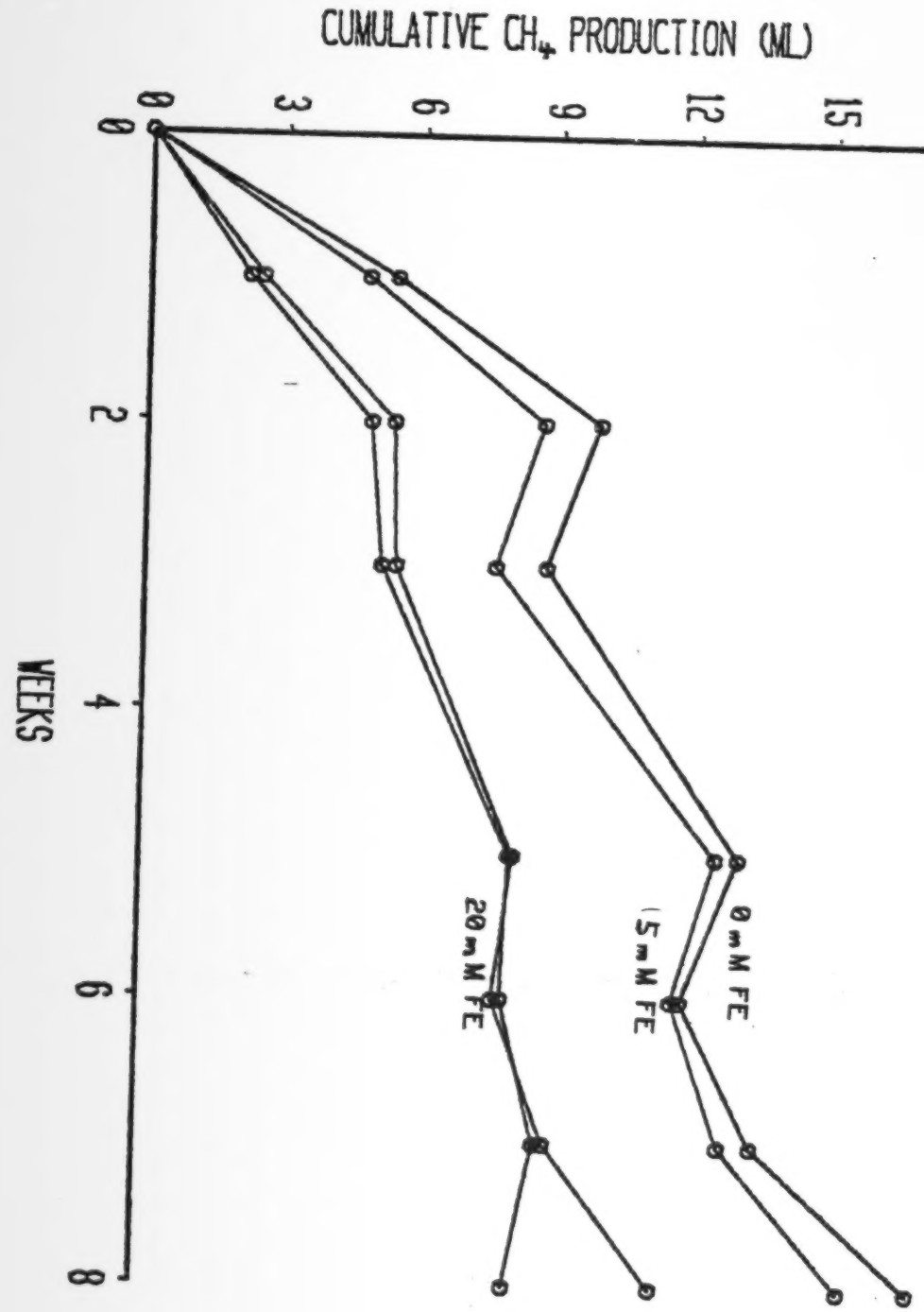


FIGURE 27 EFFECT OF IRON ON CH_4 PRODUCTION IN 10% ADRIAN SLUDGE

FIGURE 28 EFFECT OF IRON ON CH_4 PRODUCTION IN 10% JACKSON SLUDGE

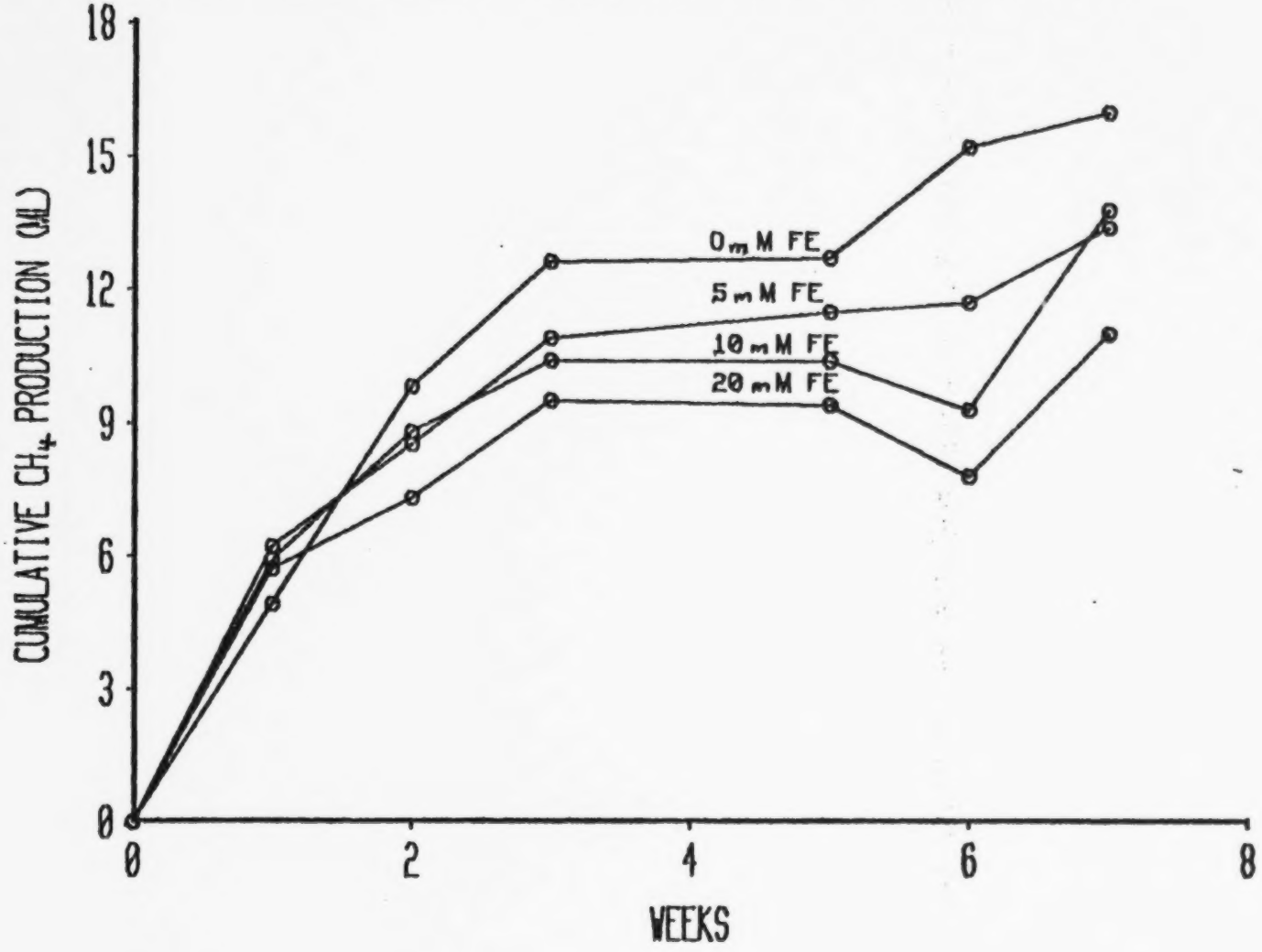


Figure 29. Effect of iron additions on soluble minerals and pH in 10% Adrian sludge.

Iron addition (uM)	Fe (uM)	P (mM)	pH
20	250	0.07	6.1-6.2
5	1.9	3.9	6.8-6.9
20[a]	290	0.08	6.1-6.2
0	0.39	>4.0	7.0-7.1

[a]Accidently doubled 10 mM Fe addition

Figure 30. Comparison of mineral salts and metals in anaerobic media vs. 10% sludge.

	Our enrichment medium (O.E.M.)	ASTM medium	Survey of anaerobic media	Survey of sludges Range	Median	Mean
Minerals (mM)						
K	6.0	3.5	2.0-36.2	0.02-2.8[a]	0.3	0.5
NH_4^+	10.0	4.3	3.2-42.4	0.03-15.4	0.4	2.1
PO_4^{2-}[b]	4.0	0.3	0.3-23.6	0.7-18.9	0.9	4.4
Ca	0.5	0.3	0.05-1.0	1.9-20.5	5.0	5.9
Mg	0.5	1.8	0.05-1.8	0.05-2.3	0.8	1.0
Fe	0.1	1.85	0.007-1.85	0.07-11.2	0.9	1.2
S^{2-}	0.5	2.0	--	--	--	--
Metals (uM)						
Mn	2.53	101.0	0.15-101.0	4.0-530	21	30
Zn	0.37	14.7	0.35-15.4	7.0-1,740	120	210
Cu	0.22	17.2	0.06-20.1	5.0-650	60	90
Co	2.10	126.0	0.42-126	0.2-1.2	0.5	0.6
Ni	0.21	--	0.08-0.21	0.1-240	6.0	30
B	0.81	97.1	0.81-97.1	5.0-280	14.0	37
Mo	0.04	41.7	0.04-41.7	1.0-1.3	1.3	1.2
Se	0.29	--	0.29-119.4	--	--	--
$NaHCO_3$	1.2 g/l	2.64 g/l				
	10% CO_2/90% N_2	30% CO_2/70% N_2				
Resazurin	1 mg/l	1 mg/l				

[a]Calculations based on a median total solids of 4.1%.

[b]Total phosphorous

Figure 31. Mineral and metal concentrations in 10% sludge.

	Source of Sludge			
Minerals (mM)	Holt	Ionia	Jackson	Mason
K	0.25	0.16	0.45	0.22
Total P	3.13	6.03	9.32	3.60
Ca	1.67	1.83	3.68	3.37
Mg	0.17	0.72	1.97	1.01
Fe	0.65	0.56	4.39	1.66
Metals (uM)				
Mn	43.4	14.7	66.1	18.0
Zn	47.5	75.3	738.7	28.1
Cu	22.5	25.5	57.1	28.2
Ni	1.8	2.2	39.3	2.2
B	4.7	6.7	29.0	11.9
Mo	1.1	1.3	5.2	1.9
Cd	0.47	0.80	8.3	0.70
Cr	5.9	16.7	908.6	10.6
Pb	3.1	5.1	12.5	3.4
Total Solids (%)	2.2	3.0	5.5	2.3

Figure 32. Effect of mineral salts media on gas production in absence of added substrate by 10% sludge.

Medium	Source of sludge			
	Jackson	Holt	Mason	Ionia
		8 week incubation		
ASTM medium[a]	13.5[c]	9.6	9.9	16.2
Our enrichment medium	18.8	18.1	18.8	20.1
Supplemental medium[b]	17.9	19.0	21.2	21.6
		6 week incubation		
ASTM medium	13.8	-2.7		
Our enrichment medium	21.8	9.4		
4 mM PO_4 =	29.3	13.1		
		2 week incubation[d]		
No addition	12.1			
ASTM medium-sulphide[a]	11.5			
ASTM medium + 2 mM sulphide	6.7			
4 mM $PO_4^=$	11.9			
Our enrichment medium-sulphide	12.8			
Our enrichment medium + 0.5 mM sulphide	10.1			

[a]Added 3.6 g $NaHCO_3$/liter instead of 2.64 g $NaHCO_3$/liter

[b]6 mM PO_4, 9 mM K^+, 10 mM NH_4^+, 10 uM Co^{++}, 10% CO_2/90% N_2

[c]Gas production (ml)

[d]Average of 5 replicates

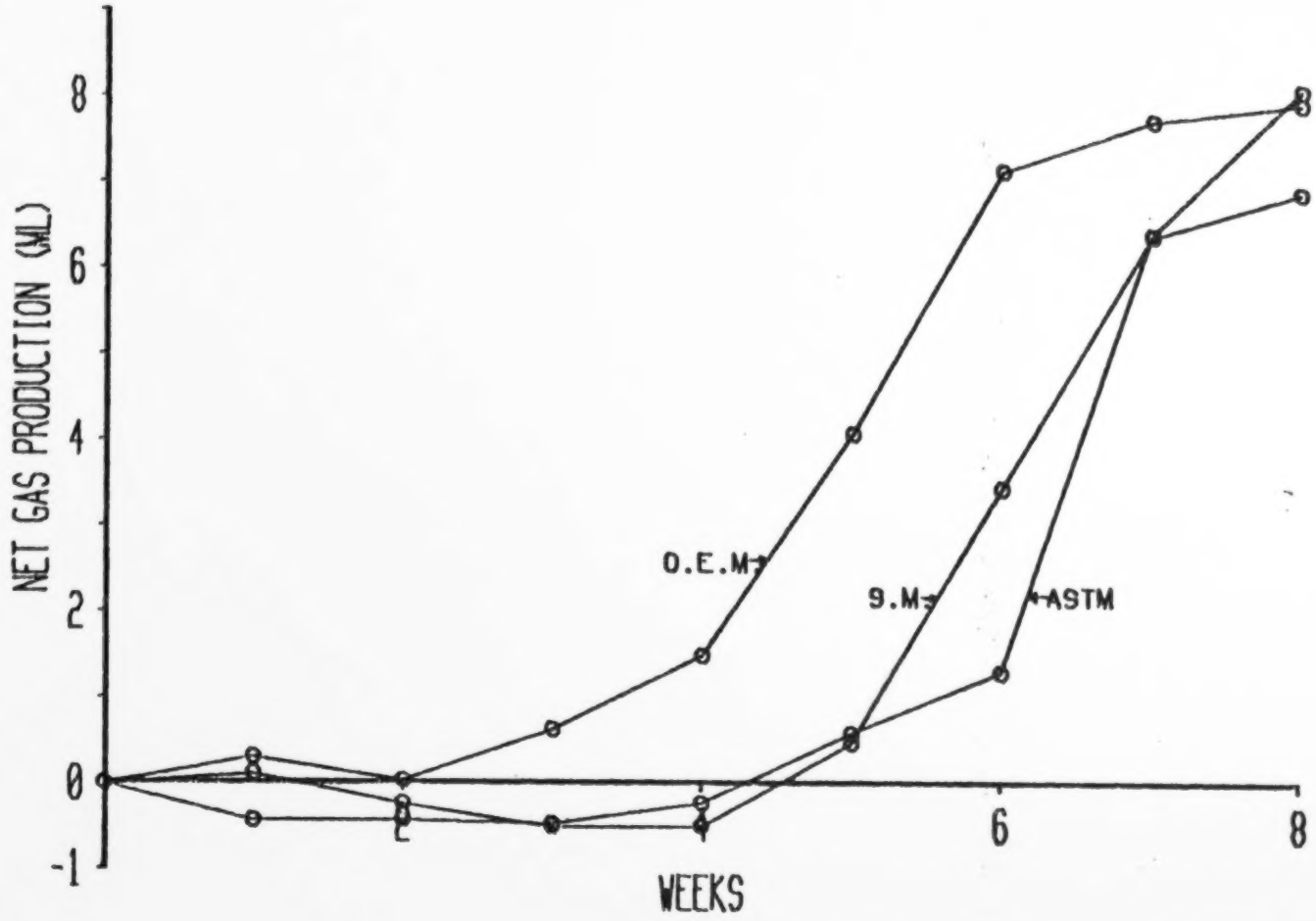
Figure 33. Effect of mineral salts media on gas production in 10% sludge.

Medium	Mean percent of theoretical gas production + S.D.			
	p-Cresol	Phthalic acid	Di-n-butylphthalate	m-Chlorobenzoic acid
		Jackson		
ASTM[a] medium	102 ± 3.2	156 ± 0	128 ± 3.9	30 ± 8.6
Our enrichment medium	108 ± 3.9	130 ± 5.0	101 ± 9.7	0
Supplemental medium[b]	79 ± 7.9	85 ± 7.7	89 ± 14.8	0
		Holt		
ASTM medium	106 ± 10.3	145 ± 19.0	59 ± 0	153 ± 6.1
Our enrichment medium	104 ± 4.5	112 ± 13.7	46 ± 3.2	101 ± 3.5
Supplemental medium	89 ± 4.6	96 ± 2.9	19 ± 5.4	65 ± 5.2
		Ionia		
ASTM medium	127 ± 7.1	183 ± 9.5	117 ± 4.7	50 ± 9.4
Our enrichment medium	115 ± 9.5	118 ± 11.4	72 ± 3.6	15 ± 10.4
Supplemental medium	99 ± 6.7	104 ± 24.8	77 ± 16.7	0
		Mason		
ASTM medium	114 ± 36.0	139 ± 13.7	111 ± 14.2	37 ± 8.5
Our Enrichment medium	96 ± 5.0	98 ± 7.2	70 ± 3.2	22 ± 8.9
Supplemental medium	104 ± 0.9	108 ± 24.7	93 ± 2.4	0

[a]Added 3.6 g $NaHCO_3$/liter instead of 2.64 $NaHCO_3$/liter

[b]6 mM $PO_4^=$, 9 mM K, 10 mM NH_4^+, 10 uM Co, 10% CO_2/90% N_2 headspace

FIGURE 34. EFFECT OF MINERAL SALTS MEDIA ON DEGRADATION OF P-CRESOL IN 10% HOLT SLUDGE



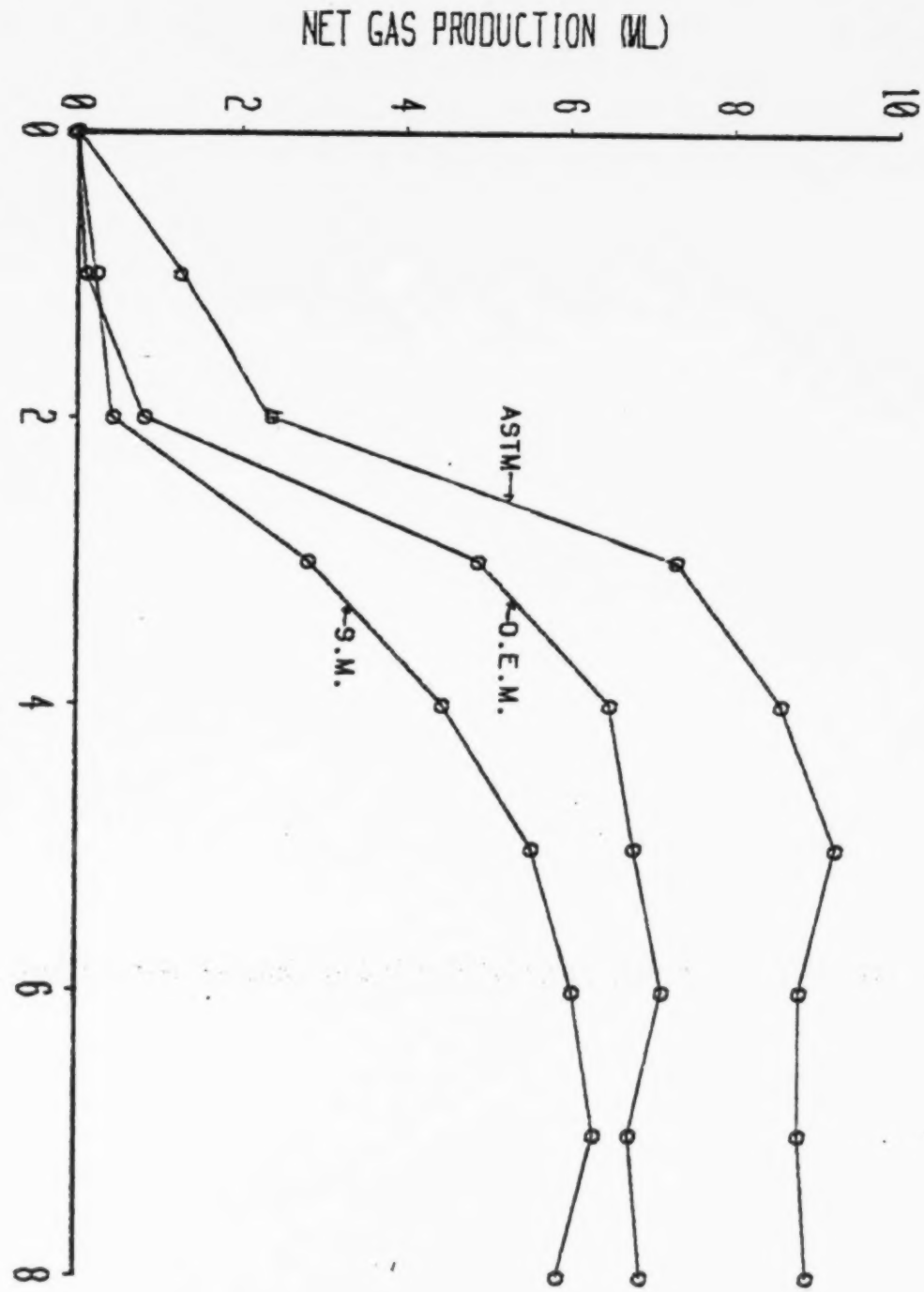


FIGURE 35. EFFECT OF MINERAL SALTS MEDIA ON DEGRADATION OF PHTHALIC ACID IN 10 HOLT SLUDGE

FIGURE 36. EFFECT OF MINERAL SALTS MEDIA ON DEGRADATION OF DI-N-BUTYLPHTHALATE IN 10% HOLT SLUDGE

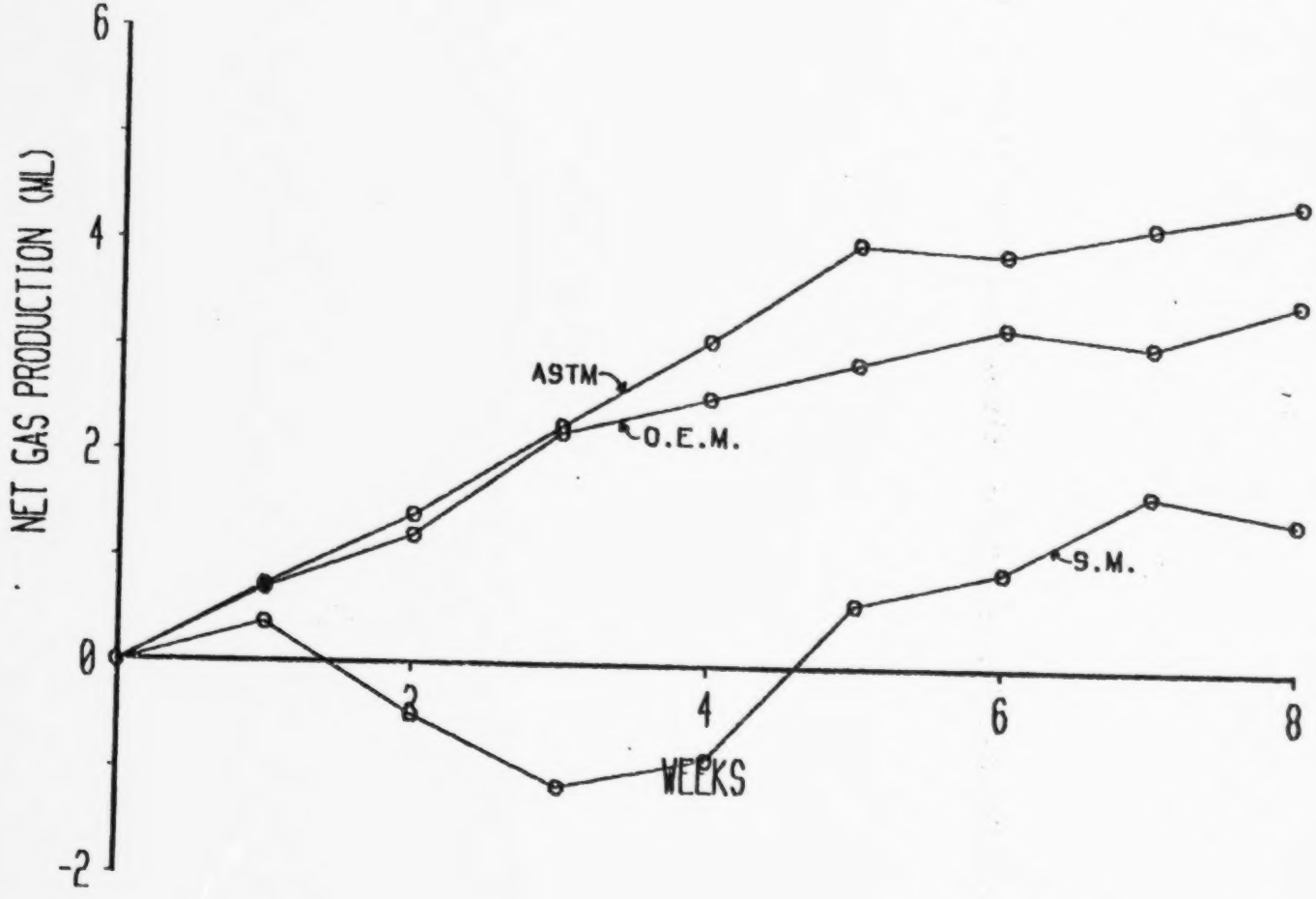


FIGURE 37. EFFECT OF MINERAL SALTS MEDIA ON DEGRADATION OF M-CHLOROBENZOIC ACID IN 10% HOLT SLUDGE

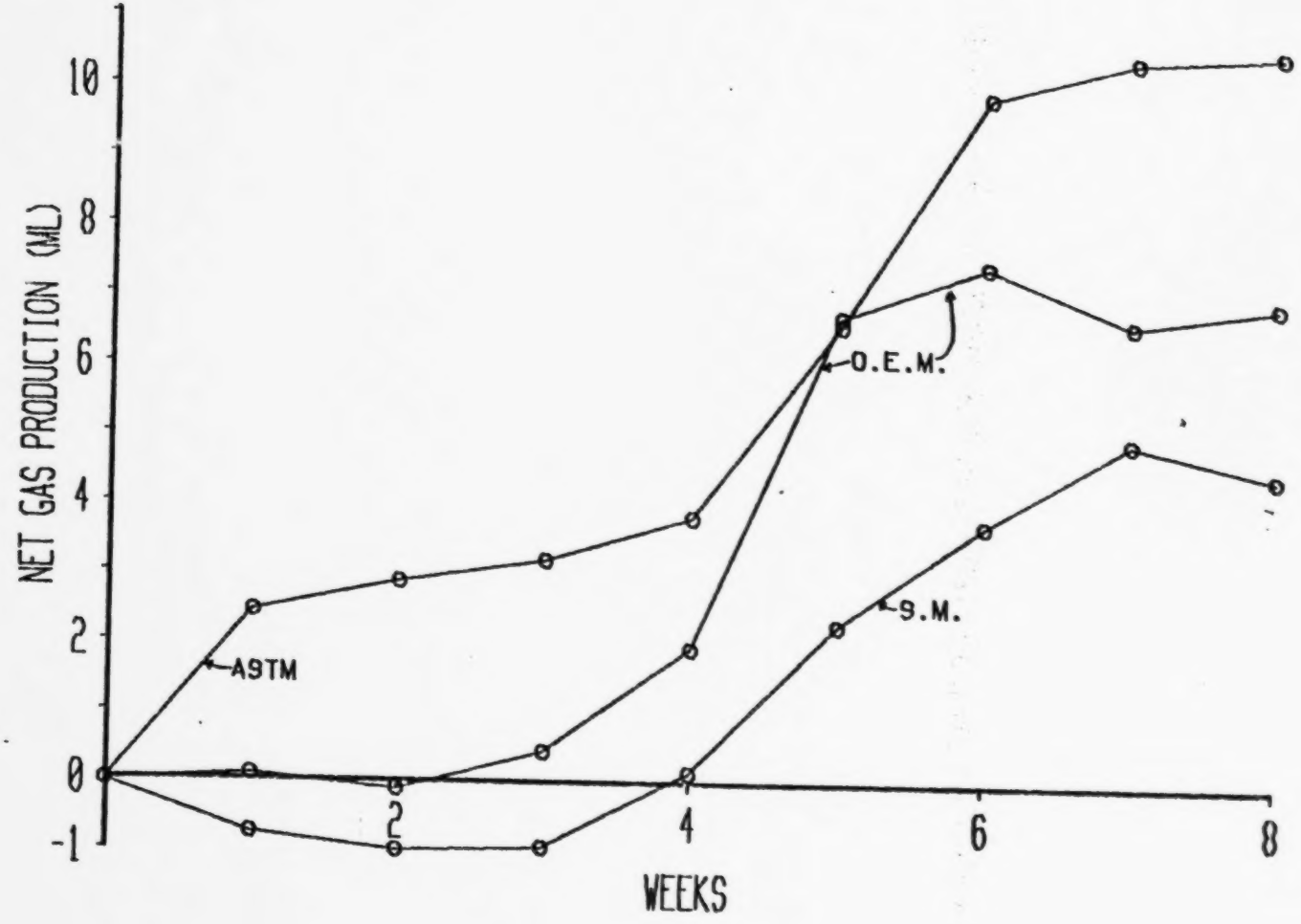


FIGURE 38. EFFECT OF MINERAL SALTS MEDIA ON DEGRADATION OF P-CRESOL IN 10% IONIA SLUDGE

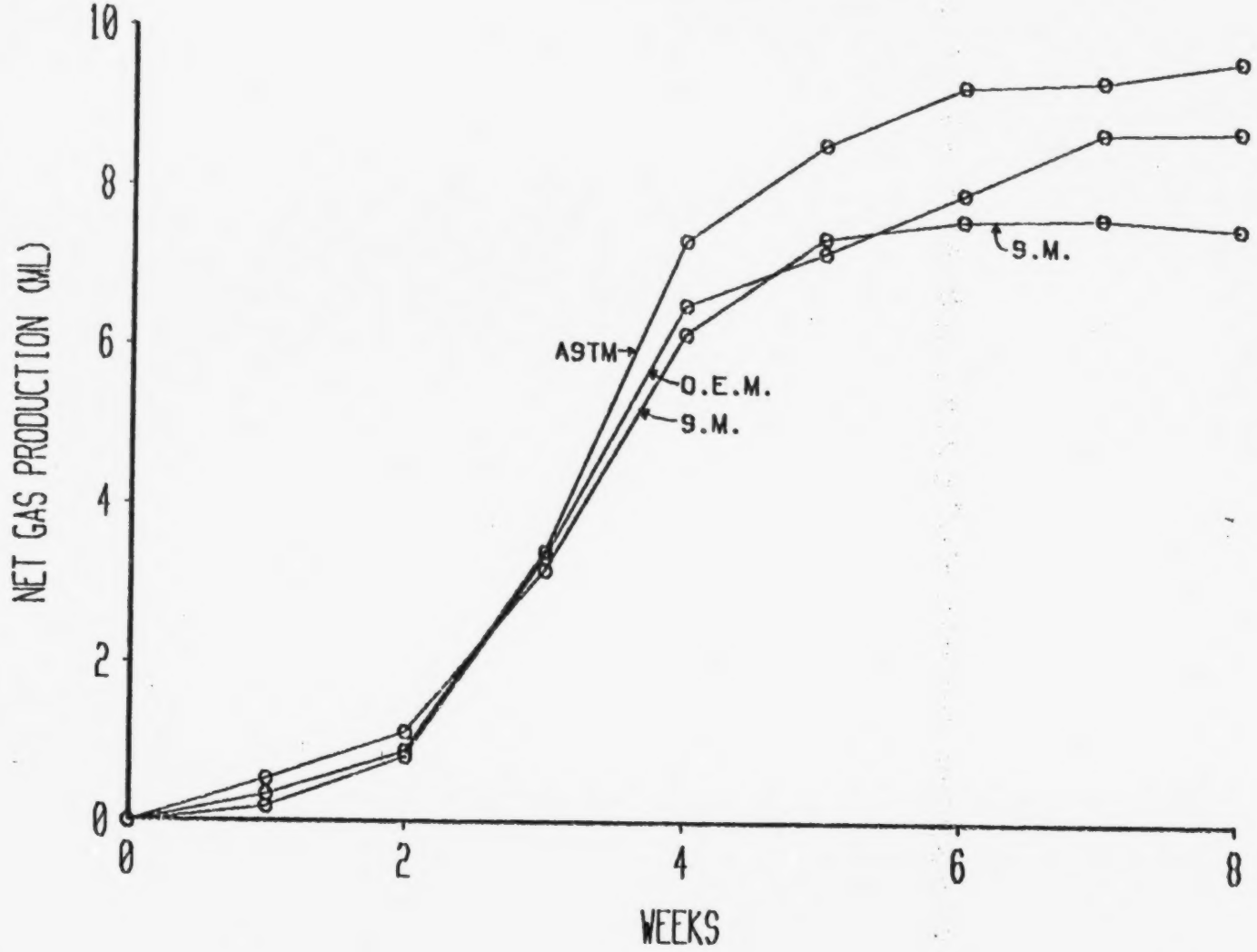


FIGURE 39. EFFECT OF MINERAL SALTS MEDIA ON DEGRADATION OF PHTHALIC ACID IN 10% IONIA SLUDGE

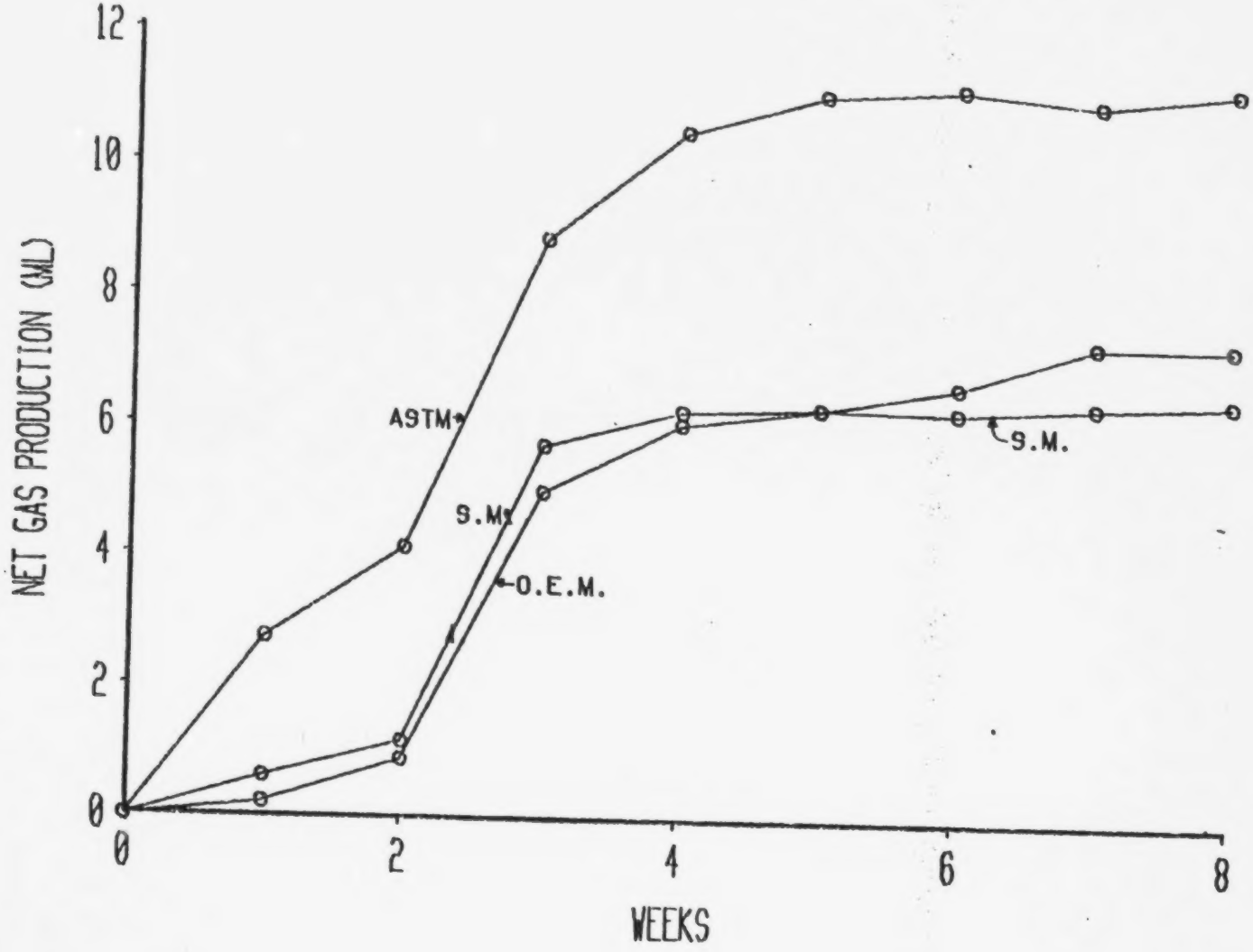


FIGURE 10. EFFECT OF MINERAL SALTS MEDIA ON DEGRADATION OF DI-N-BUTYLPHTHALATE IN 10% IONIA SLUDGE

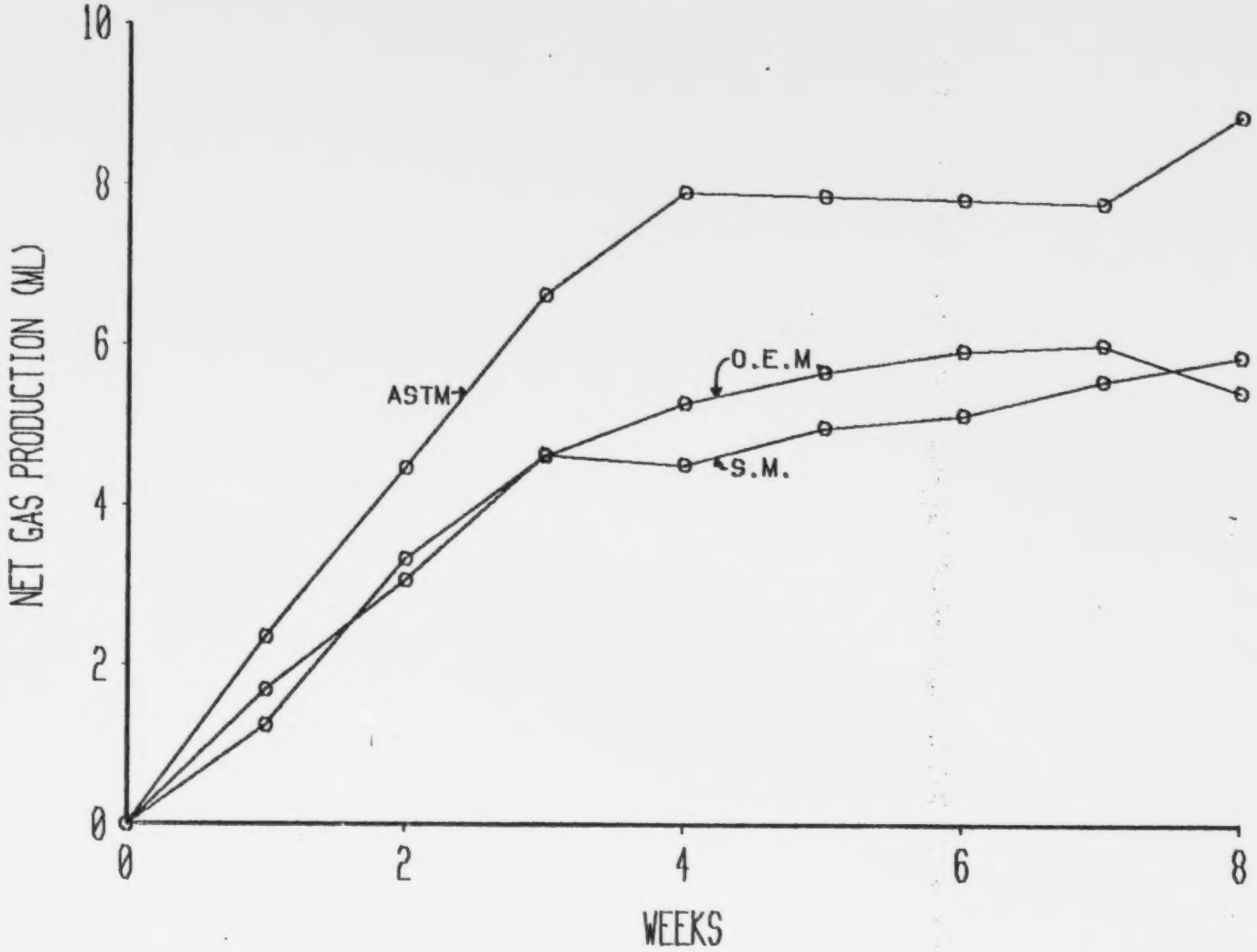
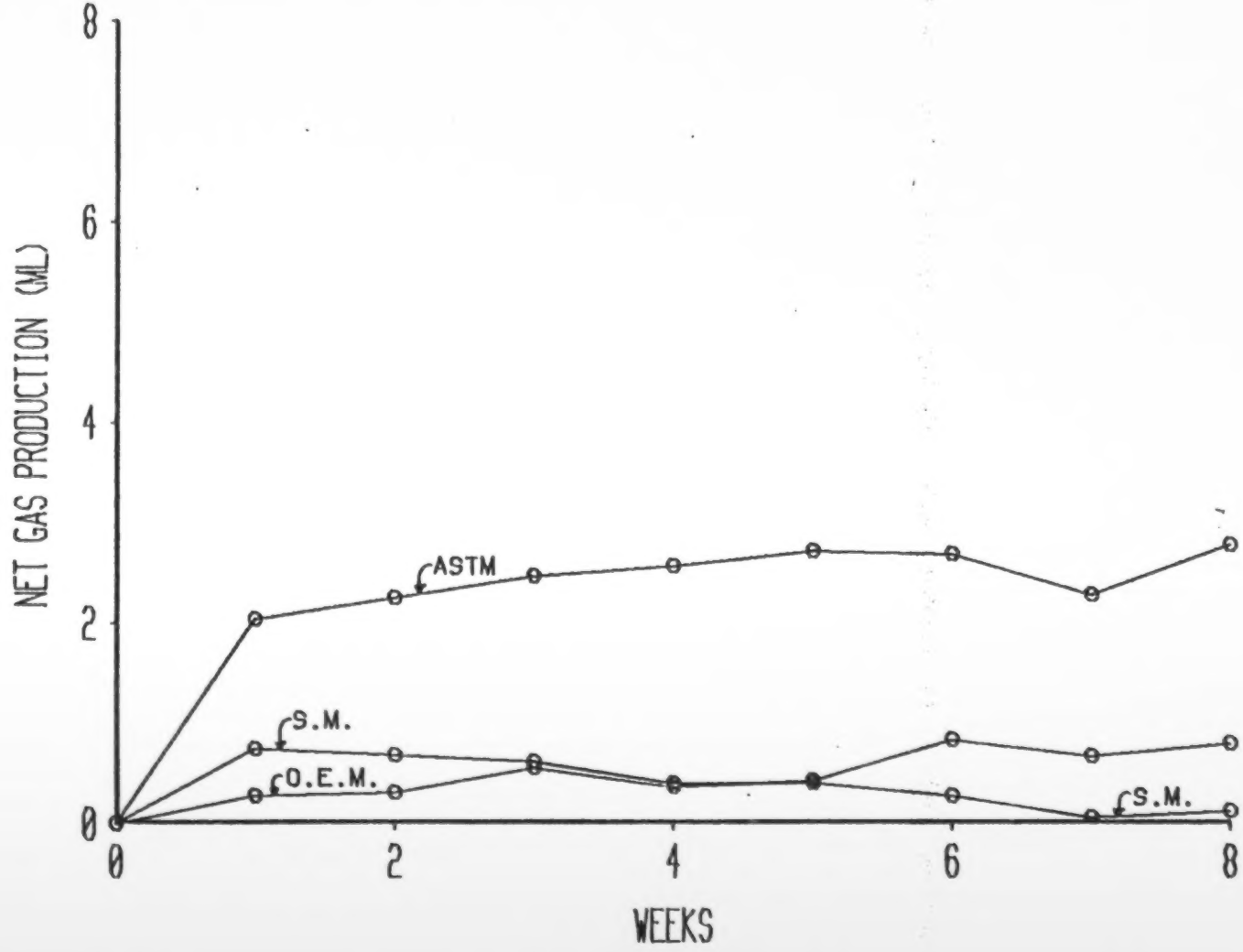


FIGURE 41. EFFECT OF MINERAL SALTS MEDIA ON DEGRADATION M-CHLOROBENZOIC ACID IN 10% IONIA SLUDGE



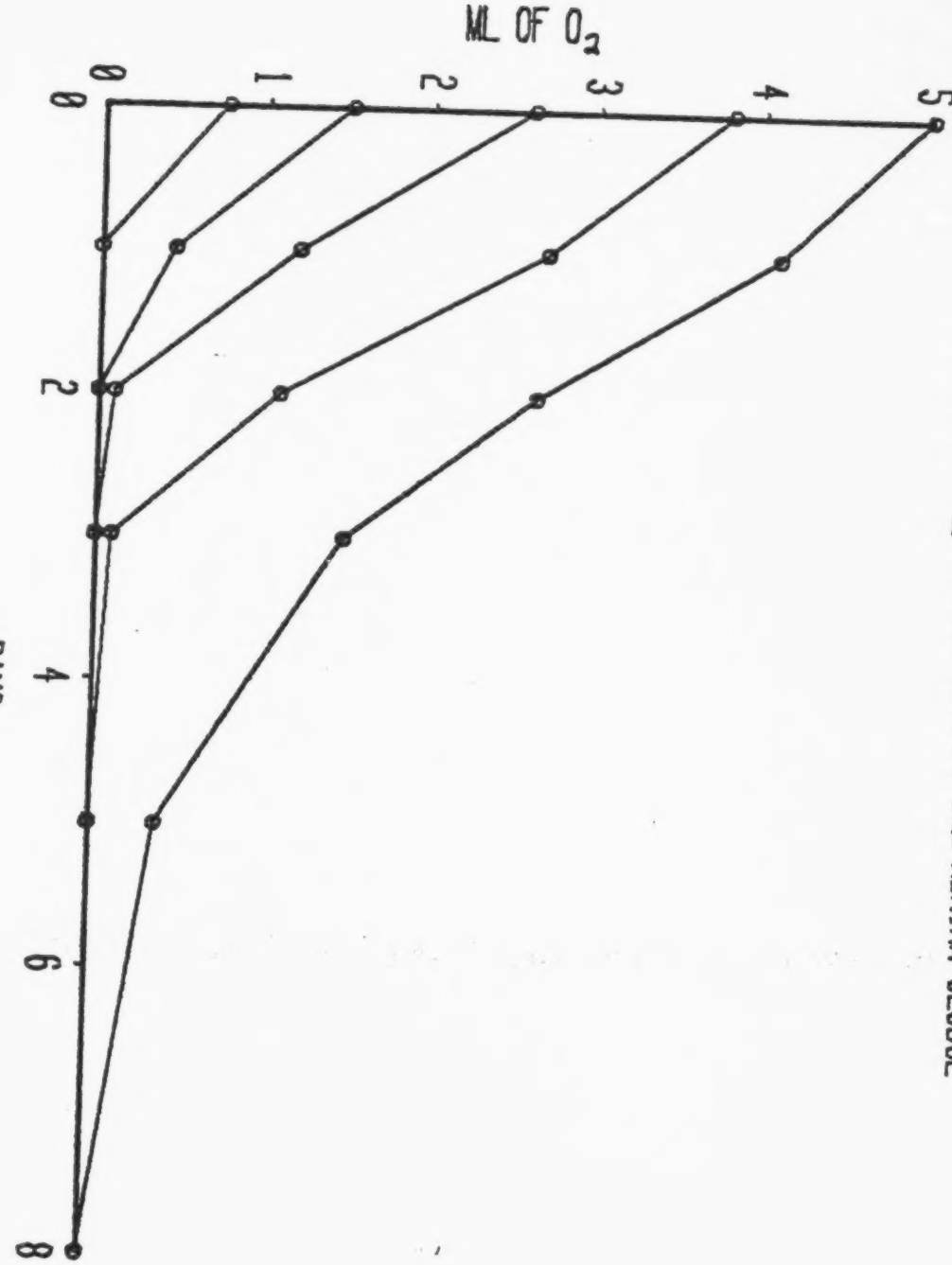


FIGURE 42. O_2 CONSUMPTION BY 10% ADRIAN SLUDGE

FIGURE 43 EFFECT OF O_2 ON GAS PRODUCTION IN 10% HOLT SLUDGE

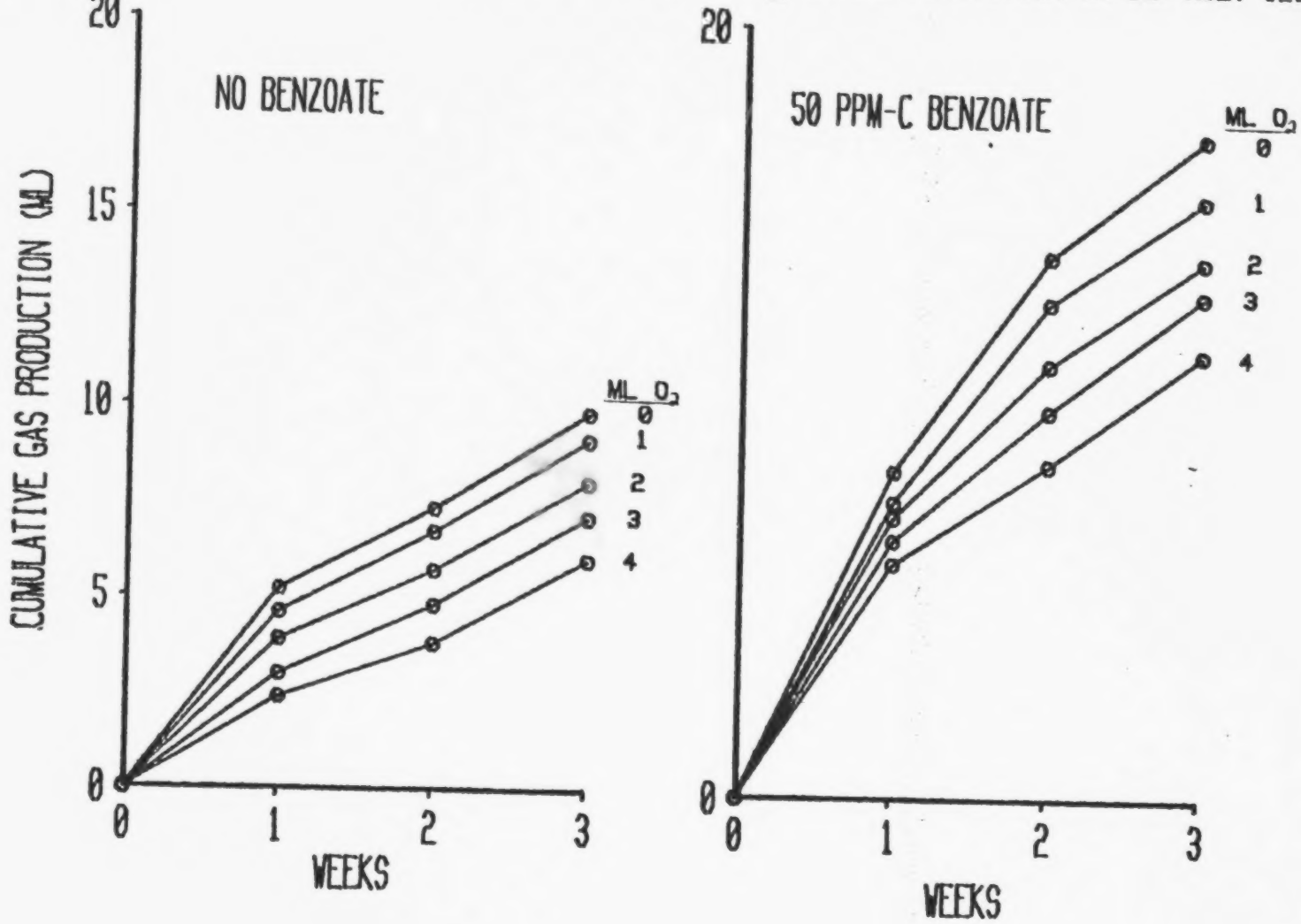


Figure 44. Effect of substrate concentration on gas production in 10% Jackson sludge

Substrate concentration (ppm carbon)	Mean percent of theoretical degradation + S.D.			
	Phenol	p-Cresol	Benzoate	Phthalic acid
25	100 ± 9.9	98	92 ± 18.0	105 ± 4.8
50	104 ± 8.0	86 ± 13.5	92 ± 6.0	104 ± 18.7
100	106 ± 6.3	98 ± 5.5	96 ± 4.7	109 ± 3.7
200	113 ± 3.1	95 ± 4.5	98 ± 3.6	100 ± 1.5
Background gas production (ml)	31.4	31.6	24.5	28.4
Theoretical gas production from 50 ppm carbon (ml)	7.4	7.6	6.5	5.7

FIGURE 45. STANDARD CURVE FOR MEASURING GAS PRODUCTION IN SERUM BOTTLES USING PRESSURE TRANSDUCER

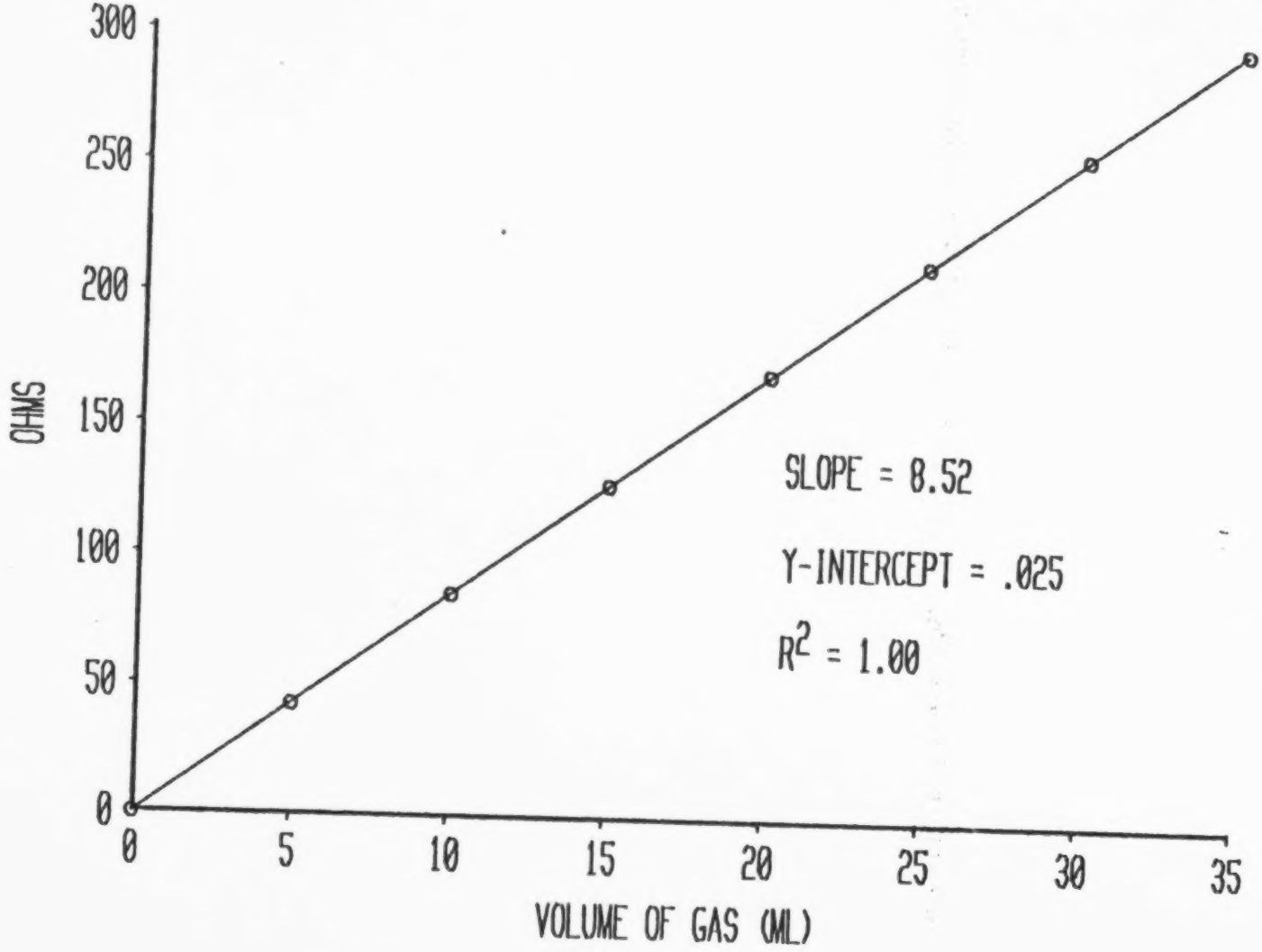


Figure 45. Reproducibility of results with different batches of sludge for several compounds and sludges.

	No. of times degradation observed / No. of times degradation tested					
Sludge	p-Cresol	Phthalic acid	m-Chlorobenzoic acid	Di-n-butylphthalate	Phenol	Benzoic acid
Jackson	5/5	5/5	1/3	5/5	2/2	2/2
Adrian	3/3	3/3	2/3	3/3		
Holt	3/3	3/3	2/2	2/2		
Mason	2/2	2/2	0/2	1/2		
Ionia	2/2	2/2	0/2	2/2		
Chelsea	2/2	2/2		1/2		

Figure 47. Effect of solvents on CH_4 and gas production from 1 mM glucose in 10% Jackson sludge.

Solvent[a]	CH_4	Gas pressure
	(% of unamended control)	
Acetonitrile	96[b]	98
Aniline	109	111
Chloroform	<0.01	33
Cyclohexane	0.03	45
Dioxane	102	93
DMSO	98	157
Ether	0.03	65
Hexane	60	99
Methylene chloride	0.01	23
Pentane	74	143
Pyridine	106	95
Toluene	0.02	34
Xylene	0.01	33

[a]Tested at concentration of 0.1% v/v

[b]Average of two replicates.